Early *in vivo* Aldosterone-induced Gene Product Usp2-45 Regulates Epithelial Sodium Channel (ENaC) by Deubiquitylation

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Abbreviations

11-β-HSD-2	11-β-hydroxysteroid dehydrogenase type 2
AIP	aldosterone-induced proteins
ASDN	aldosterone-sensitive distal nephron
Atf3	activating transcription factor 3
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic AMP
(C)CD	(cortical) collecting duct
CFTR	cystic fibrosis transmembrane conductance regulator
CNS	central nervous system
CNT	connecting tubule
Crem	cAMP responsive element modulator
cRNA	copy RNA
dNTP	deoxynucleotide-triphosphate
DCT	distal convoluted tubule
DTT	dithiothreitol
DUB	de-ubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase
EDTA	ethylenediaminetetraacetic acid
ENaC	epithelial Na^+ channel
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
GFR	glomerular filtration rate
GR	glucocorticoid receptor
h	hour(s)
HECT	homologous to E6-AP carboxyterminal
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
IVT	in vitro translation/translated
kb	kilobase
kDa	kilo Dalton
MCD	medullary collecting duct
min	minute(s)
MOPS	3-(N-morpholino) propanesulphonic acid
MAPK	mitogen-activated protein kinase
MR	mineralocorticoid receptor
Nav	voltage-gated Na^+ channel
Nedd	neural precursor cell expressed, developmentally down-regulated
NHE	Na^+/H^+ exchanger
NMDG	N-methyl-D-glucamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHA1	pseudohypoaldosteronism type 1

PI3-K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PT	proximal tubule
PVDF	polyvinylidine difluoride
PY	motif PPxY or LPxY sequence
RAAS	renin-angiotensin aldosterone system
RT	room temperature
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecyl sulphate
SE	standard error
SEM	standard error of mean
Sgk	serum and glucocorticoid-regulated kinase
Slc	solute carrier family
TAE	Tris-Ac-EDTA
TAL	thick ascending limb
TEMED	N, N, N', N'-tetramethylethylene diamine
TGFβ	transforming growth factor-β
Ub	ubiquitin
UBP	ubiquitin-specific processing enzyme
UCH	ubiquitin carboxyl terminal hydrolase
Usp	ubiquitin-specific proteases
WW1, 2, 3 or 4	WW domain 1, 2, 3 or 4

Abstract

The epithelial Na^+ channel (ENaC), located in the apical membrane of renal aldosterone-responsive epithelia, plays an essential role in controlling the Na^+ balance of extracellular fluids and hence blood pressure. Moreover, ENaC is the only Na^+ transport protein for which genetic evidence exists for its involvement in the genesis of both hypertension (Liddle's syndrome) and hypotension (pseudohypoaldosteronism type 1) revealing that the regulation of ENaC is of fundamental importance.

The final adjustment of NaCl reabsorption under the control of the mineralocorticoid hormone aldosterone takes place in the principal cells of the collecting duct in the distal nephron of the kidney. The stimulatory action of aldosterone on Na^+ reabsorption and K^+ secretion can be arbitrarily divided in an early regulatory phase, which can be observed within 30 min after initiating a hormonal treatment, and a late anabolic phase. The early actions are thought to be mediated mostly by the induction/repression of proteins belonging to the regulatory pathways that control the function of pre-existing transport effectors such as ENaC (apical entry of Na^+), the Na^+,K^+-ATPase (basolateral extrusion of Na^+) and K^+-channels.

For many years the only regulatory proteins that had been identified and experimentally shown to have an impact on ENaC function were K-Ras2, in amphibian cells, and the serum and glucocorticoid-regulated kinase Sgk1 that is also regulated in mammalian kidney.

The main focus of my doctoral thesis was to identify in the aldosterone sensitive distal nephron *in vivo*, new early aldosterone-induces gene products in particular encoding regulatory proteins other than Sgk1.

An important mechanism by which SGK1 stimulates ENaC cell-surface expression and function is by phosphorylating the ubiquitin-protein ligase Nedd4-2 and thereby interfering with the ubiquitylation of ENaC and controlling its cell-surface expression or endosomal/lysosomal degradation. Nedd4-2 binds to the PY motif in the COOH-termini of ENaC subunits and ubiquitylates ENaC on lysine residues situated in the NH_2 termini, thereby inducing channel downregulation at the plasma membrane. The short signaling cascade leading from Sgk1 induction via

inhibition of Nedd4-2 to the activation of ENaC represents so far the first direct link between the aldosterone-regulated transcriptional activity of the mineralocorticoid receptor and the activation of ENaC that has been demonstrated.

In this present study we established a new *in vivo* strategy involving Affymetrix® GeneChip® technique, with which we identified new early aldosterone-regulated mRNAs in microselected mouse distal nephron of control and aldosterone treated mice. From the 22 mRNAs that displayed a ≥ 2-fold change, 13 were downregulated. The later ones included a putative G0/G1 switch gene, a polyspecific organic anion transporter (Slc22a8) and the colonic H^+,K^+-ATPase. The 9 upregulated mRNAs included besides Sgk1 the ubiquitin protease Usp2-45 components of the TGFβ pathway and members of the CREB family of transcription factors (Grem2, Atf3 and Crem).

We showed, using expression in Hek293 cells, *Xenopus* oocytes and mpkCCD$_{c14}$ cells, that Usp2-45 deubiquitylates ENaC and stimulates ENaC-mediated sodium transport, an effect that is not additive to that of Sgk1. Furthermore, we show both that the disruption of ENaC PY motifs and the disruption of ENaC lysine residues potentially targeted by Usp2-45 prevent the action of Usp2-45 on ENaC function. These results also strongly support the hypothesis that Usp2-45 acts on ubiquitin moieties ligated onto ENaC by the ubiquitin ligase Nedd4-2. The Affymetrix screen presented in this work revealed that approximately 26 different Usp's are expressed in the aldosterone target cells of the mouse kidney tubule, but only the RNA of Usp2-45 was significant differentially regulated more than 2-fold.

In summary, our list of early aldosterone-regulated gene products contains two induced elements that interfere with the ubiquitylation of ENaC. Besides Sgk1 we identified Usp2-45 that we show to deubiquitylate ENaC in expression systems and thus represent a first example of a hormonally controlled deubiquitylating enzyme acting on a channel.

Zusammenfasssung

In der Niere ist der epitheliale Na^+-Kanal (ENaC) in der apikalen Membran der Aldosteron-sensitiven Epithelzellen lokalisiert. Dieser Kanal spielt eine wesentliche Rolle für die Kontrolle der Na^+-Homöostase und des Extrazellulärvolumens und ist folglich an der langfristigen Regulation des Blutdruckes maßgeblich beteiligt. Darüber hinaus ist bekannt, dass Mutationen von ENaC ursächlich für die Entstehung der arteriellen Hypertonie (Liddle-Syndrom) als auch der Hypotonie (Pseudohypoaldosteronismus Typ 1) beim Menschen sein können. Diese Zusammenhänge veranschaulichen die zentrale Bedeutung der exakten Regulation von ENaC unter physiologischen und pathophysiologischen Bedingungen.

Die bedarfsgerechte Feineinstellung der NaCl-Reabsorption in der Niere steht unter der Kontrolle des Mineralokortikoidhormons Aldosteron und wird durch die Hauptzellen des Sammelrohres im distalen Nephron bewerkstelligt. Der stimulierende Effekt des Aldosterons auf die Na^+-Reabsorption und K^+-Ausscheidung kann in eine frühe regulatorische Phase, die innerhalb von 30 Minuten nach Aldosterongabe beobachtet werden kann, und in eine späte trophische Phase eingeteilt werden. Es wird vermutet, dass die frühen Wirkungen von Aldosteron hauptsächlich durch die Induktion bzw. Repression regulatorischer Proteine vermittelt werden. Diese Regulatoren beeinflussen in komplexer Weise bereits vorhandene Transportproteine, wie z.B. luminale ENaC- und K^+-Kanäle und basolaterale Na^+,K^+-ATPasen.

Für viele Jahre waren die einzigen Aldosteron-induzierten regulatorischen Proteine, die man identifiziert hatte und für die experimentell eine Wirkung auf die ENaC-Funktion nachgewiesen werden konnte, K-Ras2 und die Serum- und Glukokorticoid-regulierte Kinase Sgk1.

Ein wichtiger Mechanismus, durch welchen Sgk1 die Zelloberflächen-Expression und Funktion von ENaC stimuliert, besteht in der Sgk1-abhängigen Phosphorylierung der Ubiquitin-Ligase Nedd4-2. In nicht-phosphoryliertem Zustand bindet Nedd4-2 an ein sogenanntes PY Motiv im COOH-Terminus der ENaC Untereinheiten und ubiquitinyliert ENaC an Lysin-Resten, die sich im NH2-

Terminus befinden. Die Ubiquitinylierung von ENaC leitet dann dessen Entfernung aus der Plasmamembran und die endosomale/lysosomale Degradation ein. Die kurze Signalkaskade, die von der gesteigerten Genexpression der Sgk1 über die Hemmung von Nedd4-2 zu einer Vermehrung aktiver ENaC-Kanäle in der Membran führt, stellte bis vor kurzem das wichtigste bekannte Bindeglied zwischen der transkriptionellen Aktivität des Mineralokortikoidrezeptors und der Aldosteron-induzierten Erhöhung der ENaC-Funktion dar.

Das Hauptaugenmerk meiner Doktorarbeit galt der Identifizierung neuer, unbekannter Aldosteron-induzierter Genprodukte, die wie Sgk1 im Aldosteron-sensitiven distalen Nephron unter *in vivo* Bedingungen den Salztransport maßgeblich regulieren.

Wir haben unter Zuhilfenahme der Affymetrix® GeneChip® Technologie eine *in vivo* Strategie etabliert, mit der neue, unbekannte und in einer frühen Phase Aldosteron-regulierte mRNAs identifiziert werden können. Als Ausgangsmaterial dienten uns mikro-selektierte, distale Nephronsegmente von unbehandelten und Aldosteron-behandelten Mäusen. Von den 22 mRNAs, die eine mehr als 2-fache Veränderung der Genexpression zeigten, waren 13 herunterreguliert: z.B. das mutmaßliche Switcher-Gen G0/G1, der polyspezifische organische Anionen Transporter (Slc22a8) oder die Kolon-Typ H^+,K^+-ATPase. Zu den neun hochregulierten mRNAs gehörten nebst Sgk1 die Ubiquitin-Protease Usp2-45, Bestandteile des TGFβ-Pathways und verschiedene Transkriptionsfaktoren (Grem2, Atf3 und Crem), die der CREB-Familie zugeordnet werden können. Interessanterweise wurde von den 26 unterschiedlichen Ubiquitin-Proteasen, die in den distalen Nephronsegmenten der Maus detektierbar waren, einzig und allein Usp2-45 nach Aldosterongabe mehr als 2-fach vermehrt exprimiert. Wir haben daher funktionelle Experimente angestellt, um eine mögliche Wechselwirkung von Usp2-45 und ENaC aufzudecken.
Nach Proteinexpression in Hek293-Zellen, in *Xenopus laevis* Oozyten und in mpkCCDc_{14}-Zellen beobachteten wir, dass ENaC Usp2-45-abhängig deubiquitinyliert wurde, was mit einer Stimulation des ENaC-getragenen Natriumtransports einherging. Wie zu erwarten, war die Wirkung von Usp2-45 auf ENaC nicht additiv zu der Wirkung von Sgk1, da schlussendlich beide Wege zu

einer Verminderung der Ubiquitinylierung von ENaC führen. Die Zerstörung des für die Ubiquitinylierung wichtigen PY-Motivs und des relevanten Lysin-Restes von ENaC führte zu einer Aufhebung der Wirkung von Usp2-45 auf die ENaC-Funktion. Diese Resultate untermauern die Hypothese, dass Usp2-45 auf die Ubiquitin-Reste wirkt, die durch die Ubiquitin-Ligase Nedd4-2 an ENaC gebunden werden.

Zusammenfassend kann man sagen, dass unsere Liste der „frühen“ Aldosteron-regulierten Genprodukte zwei Kandidaten enthält, die mit der Ubiquitinylierung von ENaC interferieren: Sgk1 führt zu einer verminderten Ubiquitinylierung durch Hemmung von Nedd4-2. Die im Rahmen der Arbeit erstmals als Aldosteron-reguliertes Gen identifizierte Ubiquitin-Protease Usp2-45 deubiquitinyliert ENaC und verhindert auf diesem Weg die Degradation von ENaC. Usp2-45 stellt damit das erste Beispiel eines hormonell gesteuerten, deubiquitinylierenden Enzymes dar, das auf einen Ionenkanal wirkt.

"Salt is born of the purest parents, the sun and the sea."

Pythagoras

Introduction

For freshwater animals the salt concentration in the body fluids is higher (hyperosmotic) than in the aqueous surroundings. This may result in the excessive entry of water into their bodies following the osmotic gradient. To counteract this, freshwater animals produce dilute urine and retain salts by active transport across the epithelia into the interstitial space. In addition, freshwater animals have evolved the remarkable ability to take up salts from their dilute environment. In lower vertebrates, such as amphibians, the organs involved in this process are the skin and the bladder while in fish the gills serve as the major osmolarity regulators. Terrestrial animals have also evolved systems to conserve salt and water: reptiles and birds have salt glands while in mammals the kidneys serve this purpose.

In contrast, for seawater animals, the body fluids are isotonic (marine invertebrates) or hypotonic (fish) in respect to the surrounding environment. Therefore, seawater fish excrete salt to regulate their fluid osmolarity.

From an evolutionary point of view the development of the kidney played a major role in the migration of vertebrates from the sea to the land [1].

1. General Aspects of Kidney Anatomy and Physiology

Mammalian kidneys are paired, bean-shaped structures, one located on each side against the dorsal inner surface of the lower back, outside the peritoneum in a mass of fatty tissue (small figure in Figure 1.). The outer functional layer, the cortex, is covered by a tough capsule of connective tissue. The inner functional layer, the medulla, sends papillae projecting into the renal pelvis that give rise to the ureters which later empty into the urinary bladder (Figure 1.).

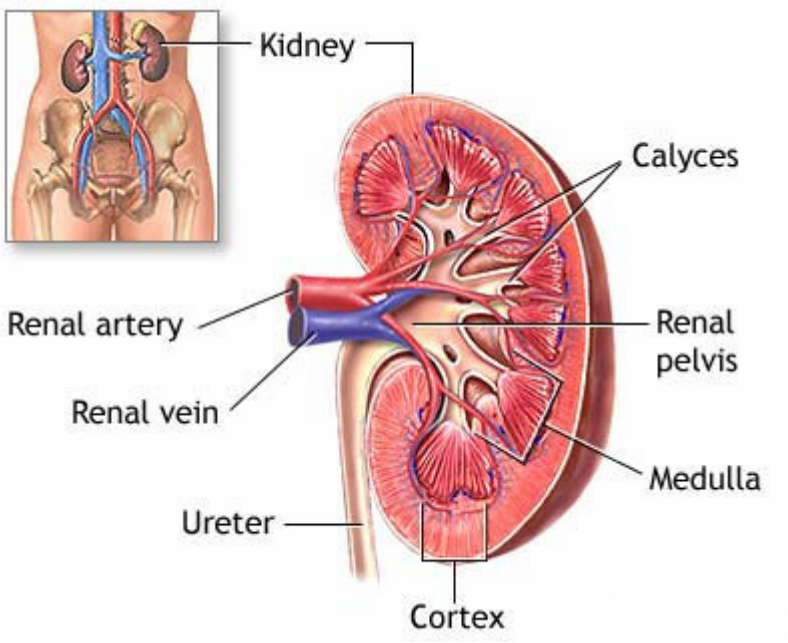


Figure 1. Schematic representation of a longitudinal section through the kidney. Adapted from www.adam.about.com.

The kidneys serve three essential functions. They work as filters, removing metabolic by-products and toxins (xenobiotics) from the blood and excreting them in the urine. They also play a key homeostatic role by regulating the body's fluid status (blood volume and blood pressure), electrolyte, amino acid and acid-base balance. And finally, the kidneys produce hormones involved in erythrogenesis, calcium metabolism and regulation of blood flow and blood pressure. To fulfill these functions the kidneys are very well supplied with blood, receiving 20-25% of the total cardiac output per minute at rest corresponding thus to the total blood volume every 4-5 minutes.

1.1. Anatomy and Functional Principles of the Nephron

Each day approximately 180 L of fluid are filtered by the human kidneys with 99 % reabsorbed such that only 1.5 to 2 liters of urine are ultimately excreted. This remarkable feature, as well as the reabsorption of water and other valuable substances is carried out by several hundred (in lower vertebrates) to many thousand (in small mammals), and a million or more nephrons (in humans and other large species). The functional unit of the mammalian kidney is the nephron, an intricate epithelial tube which is closed at its beginning and open at its distal end. Each kidney contains around one million nephrons (in humans), which empty into the collecting ducts. The collecting ducts combine to form papillary ducts, which eventually empty into the renal pelvis. At the closed end, the nephron is expanded to form a cup-shaped Bowman's capsule (Figure 2.B). The lumen of the capsule is continuous with the narrow lumen that extends through the renal tubule. A tuft of capillaries forms the renal glomerulus inside Bowman's capsule. Therein, two layers of cells are responsible for filtering from the blood a wide range of high molecular compounds including proteins as small as 1-4 nm. The blood passes through the single-cell layer of the capillary walls, through a basement membrane, and finally enters the Bowman's capsule through a single-cell layer of epithelium. The resulting ultrafiltrate or "primary urine" contains approximately the same concentration of electrolytes, sugars, amino acids etc. as is found in the plasma.

From the Bowman's capsule the filtrate enters the renal tubule where the selective reabsorption of water, inorganic ions and other molecules from the glomerular filtrate occurs.

In terms of morphology, there exist two subtypes of nephrons; the cortical and the juxtamedullary nephrons (Figure 2.B). Cortical nephrons have their glomeruli in the outer cortex located near the kidney surface and they possess relatively short loops of Henle that extend only a short distance into the outer zones of the medulla. The juxtamedullary nephrons, in contrast, have their glomeruli in the inner part of the cortex located deeper than cortical nephrons and their long loops of Henle plunge very deep into the inner zone of the medulla. The wall of the renal tubule is one cell layer thick. This epithelium separates the lumen, which contains the ultrafiltrate, from the interstitial fluid. In the early portions of the nephron epithelial cells are morphologically specialized for a high rate of solute transport,

bearing a dense pile of microvilli on their luminal, or apical, surfaces and deep infoldings at their baso-lateral membrane.

Nephrons can be structurally and functionally divided into three main regions or segments (Figure 2.B) having distinct roles regarding the reabsorption of ions, glucose, amino acids and water.

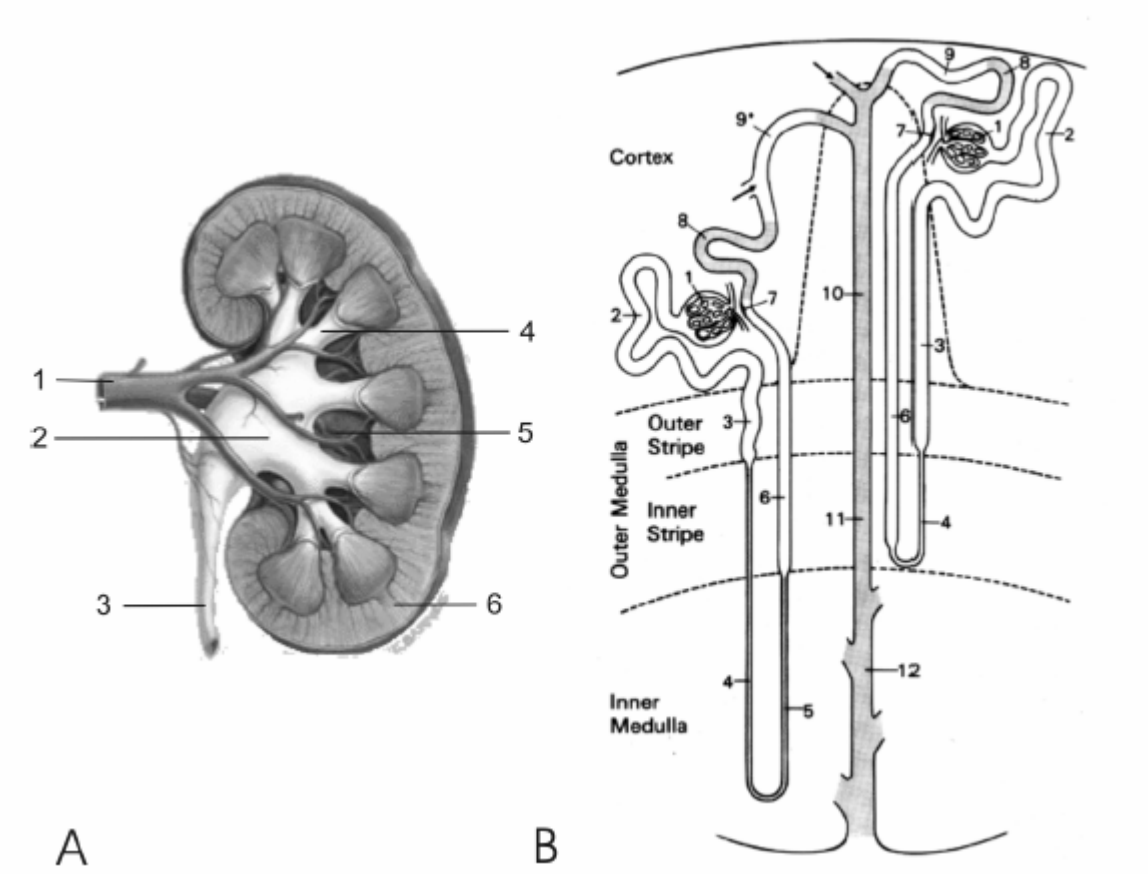


Figure 2. Panel **A**, anatomical scheme of the kidney: 1. renal artery, 2. renal pelvis, 3. ureter. 4. calyx, 5. medulla, 6. cortex; Panel **B**: schematic representation of superficial and deep nephrons. 1. glomerulus, 2. proximal tubule (PROX) 3. proximal straight tubule 4. descending thin limb of the loop of Henle (LOH). 5. ascending thin limb of the loop of Henle. 6. thick ascending limb of the loop of Henle, 7. macula densa; 8. distal convoluted tubule (DCT); 9. connecting tubule (CT); 10 cortical collecting duct (CCD); 11. outer medullary collecting duct; 12 inner medullary collecting duct. Adapted from www.umm.edu/transplant/kidney/ and Kriz and Kaissling, 1992.

The segments of the nephron are the proximal nephron (Bowman's capsule and proximal tubule), the loop of Henle (with a descending limb and an ascending limb) and the distal nephron (after the ascending limb of the loop of Henle merges into the distal tubule). Distal tubules from many nephrons merge into one unique collecting duct.

1.2. The Segments of the Nephron Tubule

Filtration of the blood requires a high blood pressure, therefore the blood is led directly from arteries via the vasa afferentia into the glomeruli (Figure 2.A and B). Plasma is filtered at the level of the glomerulus and as it flows along the nephron its composition changes dramatically. While much of the water, electrolytes, peptides, amino acids and sugars are reabsorbed by the specialized tubular epithelia, other filtrate components such as urea, xenobiotics, organic acids and bases and some NaCl, KCl, remain in tubular fluid and end up in the urine (Figure 3.). As the filtrate flows through the tubule, most filtered Na^+ and water are also reabsorbed by epithelium of the lining. Under normal conditions, less than 1% of the filtered sodium load is excreted.

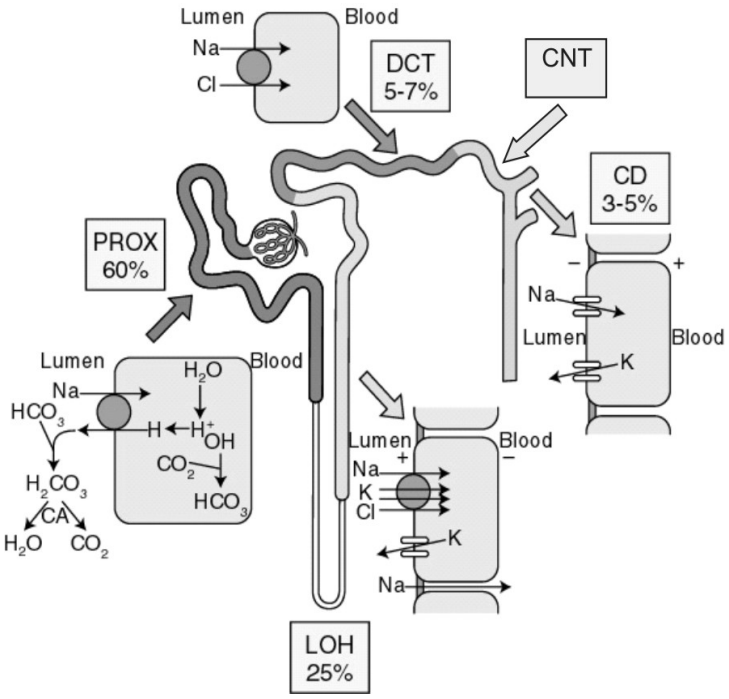


Figure 3. Na^+ reabsorption along the mammalian nephron. All Na^+ transporting cells in the nephron express the Na^+,K^+-ATPase at the basolateral membrane (not shown at this scheme for clarity). The most quantitatively important Na^+ entry pathways are shown here (NHE3-sodium/proton exchanger in the proximal tubule (PROX), furosemide-sensitive Na^+,K^+,$2Cl^-$ cotransporter in the loop of Henle (LOH), thiazide-sensitive NaCl cotransporter in the distal convoluted tubule (DCT) and amiloride-sensitive epithelial soidium channel ENaC in the connecting tubule (CNT) and cortical and medullary collecting ducts (CD).
Adapted from www.kidneyatlas.org.

Figure 3. shows an overview of the morphological segments of the nephron tubule. The following description of the morphological and physiological aspects includes sodium reabsorption from the tubular fluid into the blood.

1.2.1. The Glomerulus

The initial part of a nephron, the glomerulus, is formed by a capillary network of vessels and is responsible for the filtration of plasma. This structure is approximately 0.2 mm in diameter and acts as a coarse filter, allowing all but

cellular elements, i.e. blood cells, and plasma large molecular weight proteins to pass into the glomerular filtrate. Glomeruli are located in the kidney cortex (Figure 2.A).

1.2.2. The Proximal tubule (PT)

The proximal tubule is the initial and phylogenetically the oldest region of the tubule system. Anatomically, the PT, which begins immediately following the glomerulus, is divided into the proximal convoluted and the proximal straight tubule. However, based on ultrastructure, the proximal tubule can alternatively be subdivided into three segments: S1, S2 and S3. The S1 segment starts at the glomerulus and includes the first portion of the proximal convoluted tubule. The S2 segment starts within the second half of the proximal convoluted tubule and continues into the first half of the proximal straight tubule. Finally, the S3 segment includes the distal half of the proximal straight tubule that extends into the medulla.

Both the apical (luminal) and the basolateral (peritubular) membranes of proximal-tubule cells are extensively amplified. The apical membrane contains numerous infoldings, called microvilli, forming a well-developed brush border system. This enlargement of the apical membrane surface area correlates with the main function of this nephron segment, namely, the reabsorption of the bulk of the filtered fluid back into the circulation. In the proximal tubule, two thirds of the whole fluid is reabsorbed, along with 60-70 % of the filtered sodium, chloride, potassium, bicarbonate and phosphate. Further, important nutrients like glucose and amino acids are almost entirely reabsorbed. The basolateral membranes of adjacent proximal-tubule cells form numerous interdigitations, bringing the abundant supply of mitochondria in close contact with the plasma membrane. These organelles supply ATP to power the Na^+,K^+-ATPase pumps which transport Na^+ out of and K^+ into the cell. The interdigitations of the lateral membranes also form an extensive extracellular compartment bounded by the tight junctions at one end, and by the basement membrane of the epithelium at the other end. Proximal-tubule cells contain lysosomes, endocytic vacuoles and a well-developed system of endoplasmic reticulum. Proximal-tubule cells are also characterized by a prominent Golgi apparatus, which is important for synthesizing many membrane components, for sorting them and for targeting them to specific surface sites.

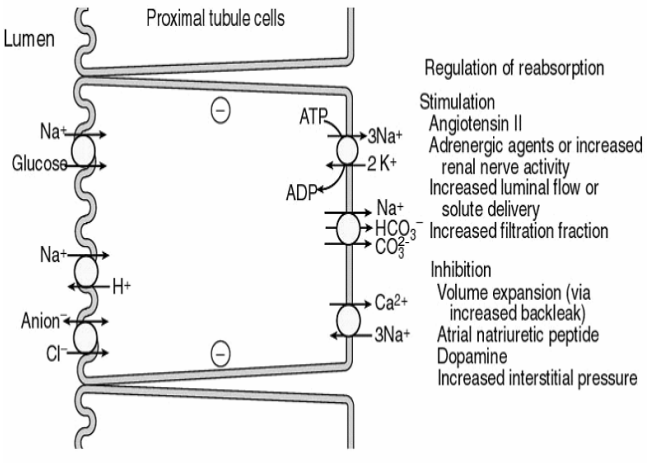


Figure 4. Cell of the proximal tubule reabsorption showing the main net entry and exit pathways for the indicated ions and glucose leading to reabsorption. Stimulatory and inhibitory hormones or mechanisms regulating these reabsorption mechanisms are listed on the right side.
Adapted from www.kidneyatlas.org.

From the S1 to the S3 segments, cell complexity progressively decreases, correlating with a gradual decline in reabsorptive rates along the tubule, thus, the cells exhibit a progressively less developed brush border system, diminished complexity of lateral cell interdigitations, a reduced basolateral cell membrane area and a decrease in the number of mitochondria.

Most of the luminal sodium entry (>80 %; Figure 3.) is directly coupled to active proton extrusion via the Na^+/H^+ exchanger NHE-3 [2] and possibly NHE-2 [3] and secondarily to bicarbonate or chloride reabsorption. Na^+ is extruded across the basolateral membrane via the HCO_3^-/Na^+ cotransporter as well as the Na^+,K^+-ATPase (Figure 4.). Luminal and basolateral membranes are freely permeable to water due to the expression of the water channel aquaporin-1 (AQP-1) [4]. Further, the existence of leaky tight junctions permits limited paracellular diffusion of water and salt ions from the lumen to the interstitial space. In the PT, the osmolarity of the fluid is not altered and therefore remains isotonic.

1.2.3. The Loop of Henle

The loop of Henle has a crucial role in concentrating the urine. The function of the loop of Henle is to produce an increasing osmotic gradient from the cortex to the tip of the renal papilla by the *counter-current multiplier* mechanism. It comprises two functionally asymmetric parts, the descending thin and the ascending thin and thick limbs (TALs). In the descending thin limb the tubular fluid undergoes an increase in osmolarity, since the water permeability of the

membrane allows the diffusion of water to the (highly osmolar) blood site. In contrast, the entire ascending limb (thin and thick regions) is water impermeable and urinary sodium is reabsorbed actively in the thick ascending limb (TAL; Figure 3.).

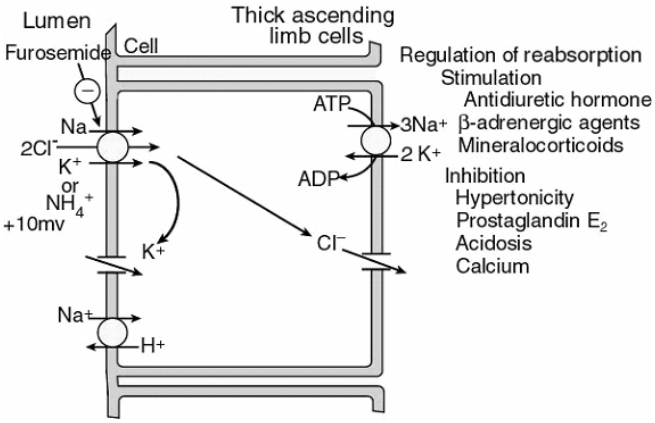


Figure 5. Main sodium and potassium transport mechanism in the thick ascending limb (TAL) of the loop of Henle involved in sodium reabsorption. The stimulatory and inhibitory hormones acting on this tubular segment and the mechanisms regulating the reabsorption mechanisms are listed on the right side. Adapted from www.kidneyatlas.org.

Critically the apical cotransport of Na^+, K^+ and Cl^- into the cells occurs in the absence of water permeability leading to the production of a hypotonic urinary fluid.

Regarding the Na^+ reabsorption (Figure 3.; ~ 25% of Na^+ load), basolaterally located Na^+,K^+-ATPase pumps generate a sodium gradient which is to a large extent, dissipated by the apical electroneutral Na^+-K^+-$2Cl^-$ cotransporter [5].

The TAL leads back to the glomerulus forming tight contact with the juxtaglomerular apparatus, allowing feedback regulation from the distal nephron to the glomerulus. The loop of Henle is only present in birds and mammals. Vertebrates which lack the loop of Henle are incapable of producing hyperosmolar urine.

1.2.4. The Juxtaglomerular Apparatus

This is a specialization of the glomerular afferent arteriole and the distal convoluted tubule in each nephron, which controls glomerular filtration rate (GFR) at the level of the individual nephron. It is involved in the regulation of systemic blood pressure via the renin-angiotensin-aldosterone mechanism. The juxtaglomerular apparatus has three components: macula densa, juxtaglomerular cells and extraglomerular mesangial cells.

The Macula densa is composed of closely packed tubular epithelial cells found at the end of the TAL. The Juxtaglomerular cells are specialized smooth muscle cells of the wall of the afferent arteriole forming a cluster around it, just before it enters the glomerulus. Finally, the extraglomerular mesangial cells are flat and elongated cells embedded in a network of mesangial matrix. Their role in the juxtaglomerular apparatus is unclear.

It is believed that the cells of the macula densa (Figure 3.) respond to NaCl concentration within the distal tubule, and in turn regulate both the diameter of the afferent arteriole and the secretion of renin by the juxtaglomerular cells. Renin is a participant in the renin-angiotensin-aldosterone system, its physiological role in the regulation of glomerular filtration, renal tubular reabsorption of Na^+ and water will be discussed later on.

1.2.5. The Distal Convoluted Tubule (DCT)

The distal convoluted tubules are located in the cortex, intertwined with the proximal convoluted tubules which are longer and then occupy more space. The distal tubules can be differentiated from the proximal tubules by the lack of a brush border membrane and their lumen is larger and more clearly defined.

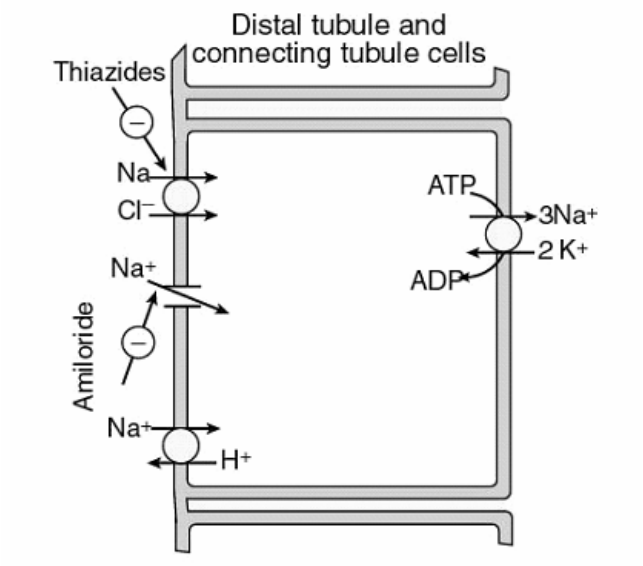


Figure 6. Mechanisms of sodium chloride reabsorption in the distal tubule. The distal tubule has predominantly a Na^+/Cl^- cotransporter, which is inhibited by thiazide diuretics. In the connecting tubule (CNT), sodium channels and a sodium-hydrogen exchange mechanism also are present. Amiloride inhibits sodium channel activity. Again the sodium-potassium ATPase (Na^+-K^+ ATPase) on the basolateral membrane provides most of the driving force for sodium reabsorption.
Adapted from www.kidneyatlas.org.

In the distal convoluted tubule, 5 - 7 % of filtered sodium is reabsorbed via a variety of sodium transport mechanisms. In the more proximal segments (DCT1) Na^+ reabsorbtion is achieved by the apical localized thiazide-sensitive Na^+-Cl^- cotransporter (NCC: also termed as TSC), whereas in the more distal (DCT2)

segments, the Na^+-Cl^- cotransporter together with the amiloride-sensitive epithelial sodium channel (ENaC) [6] form the apical Na^+ transport pathway. This second portion of the distal convoluted tubule (DCT2) along with the connecting tuble (CNT) and the collecting duct (CD) belong to the Aldosterone Sensitive Distal Nephron (ASDN).

Dietary Na^+ restriction, which is a potent stimulus of aldosterone secretion, has been demonstrated to increase thiazide-sensitive Na^+ transport in DCT [7].

1.2.6. The Connecting Tubule (CNT) and Collecting Duct (CD)

The connecting tubules join the distal convoluted tubule to the collecting duct. Tubuli of several nephrons run into the collecting duct (CD) channel system. The water permeability of the cells of the connecting tubules and of the collecting ducts is controlled by the antidiuretic hormone (ADH). The final adjustment (“fine tuning”) of sodium (but also of potassium, acid/base and water) excretion takes place at the level of the collecting duct. Accordingly, the collecting system may absorb between 3 and 5 % of the filtered sodium.

The collecting duct consists of a tight epithelium, displaying an exclusively high transepithelial electrical resistance and very low paracellular water permeability. Morphologically, the epithelia lining the collecting duct consist of two cell types; the segment-specific cells (principal cells) and the intercalated cells, which disappear in the medullary parts of the duct (Figure 7.). The latter cells have the ability to secrete and/or reabsorb protons and bicarbonates, being responsible for the regulation of the acid/base balance, whereas sodium and water reabsorption and potassium secretion originates in the principal cells.

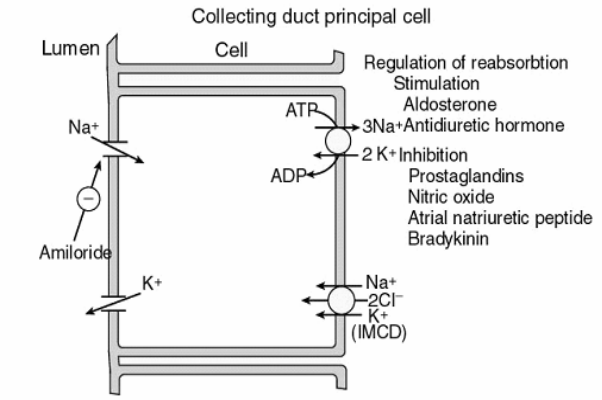


Figure 7. Schematic representation of the segment specific cell of the cortical collecting duct (CCD), showing apical and basolateral transport pathways for sodium.
Adapted from www.kidneyatlas.org/.

In the principal cells the sodium reabsorbing machinery is formed by the apical epithelial sodium channel ENaC and the basolaterally located Na^+,K^+-ATPase (Figure 3.). By extruding 3 Na^+ to the blood side in exchange for 2 K^+, the Na^+,K^+-ATPase generates the driving force for Na^+ entry via ENaC. Because the sodium reabsorption is electrogenic (both apical and basolateral transport systems), a large lumen-negative transepithelial voltage (-10 to -60 mV) is generated. As Na^+ reabsorption decreases further down the collecting duct, the transepithelial voltage decreases and turns slightly lumen positive. The main extrusion of potassium from the intracellular compartment of CCD cells to the tubular lumen is provided by ROMK, an inwardly rectifying potassium channel [8]. Potassium secretion is favored by depolarization due to apical Na^+ entry. The CCD is also important for the water reabsorption that occurs via the apical vasopressin- induced water channel aquaporin 2 (AQP-2), the constitutively expressed basolateral water channels aquaporin 3 (AQP-3) and by aquaporin 4 (AQP-4). Moreover, the basolateral membrane contains a Na^+/H^+ exchanger (NHE-1), which is involved in intracellular pH regulation [9].

In general, sodium reabsorption in the segment-specific cells of the collecting duct of the ASDN is performed mainly via amiloride sensitive sodium channels (ENaC).

1.2.7. The Ureter

The ureters are muscular tubes, which carry urine from the kidneys to the bladder. The lumen of the ureter is heavily folded and is lined with transitional epithelium.

2. The Salt/Water Homeostasis and Blood Pressure Regulation

Sodium is the predominant cation in extracellular fluid (ECF) and the volume of ECF is directly proportional to the content of sodium in the body. Impairment of sodium balance (homeostasis), therefore, may be viewed as disorders of ECF volume. The body must maintain ECF volume within acceptable limits to maintain tissue perfusion because plasma volume is directly proportional to ECF volume. The plasma volume is a crucial component of the blood volume that determines rates of organ perfusion. ECF volume is maintained within narrow limits despite wide variations in dietary sodium intake.

Goldblatt and later in the early 80's Guyton et al. [10] demonstrated the importance of the kidney in controlling the arterial pressure. Many mechanisms are involved in the acute regulation of pressure homeostasis, such as endogenous regulators of vascular tone, hormonal vasoconstrictors, neural inputs, and other nonrenal mechanisms. The long-term regulation of arterial pressure is intimately linked to the ability of the kidneys to retain/excrete sufficient sodium chloride to maintain normal sodium balance (net intake = net loss [11]), extracellular fluid volume (ECF) and blood volume at normotensive arterial pressures. Blood pressure (BP) is controlled by renal volume excretion, which is adjusted to a set point.

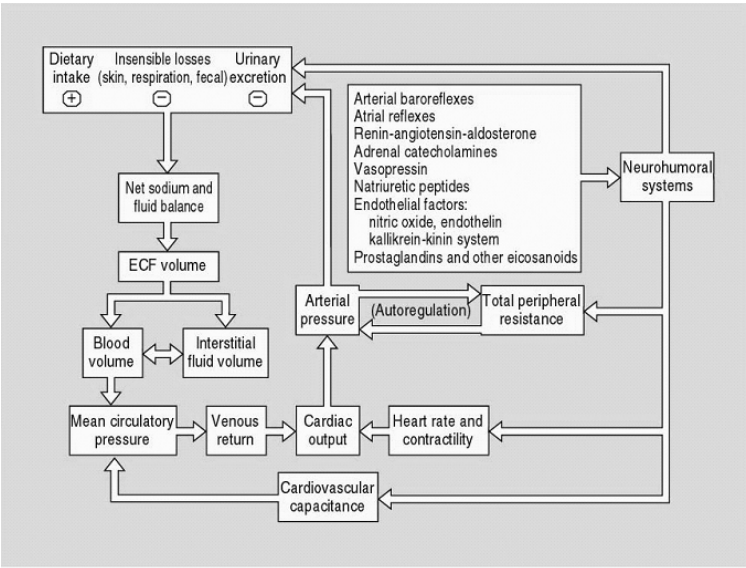


Figure 8. Volume determinants of arterial pressure. The two major determinants of arterial pressure, cardiac output and total peripheral resistance, are regulated by a combination of short- and long-term mechanisms.
Adapted from Navar [12].

In normal persons, an increased intake of sodium chloride leads to appropriate adjustments in the activity of various humoral, neural, and paracrine mechanisms (Figure 8.). These mechanisms alter systemic and renal hemodynamics and increase sodium excretion without increasing arterial pressure [12, 13].

Glomerular filtration rate (GFR), GFR-independent natriuretic factors and importantly the renin-angiotensin-aldosterone system affect sodium homeostasis.

The precise control of BP occurs via Na^+ homeostasis and involves the precise regulation of ENaC in the aldosterone-sensitive distal nephron (ASDN). This has been corroborated by the linkage of mutations in the genes encoding ENaC subunits and Liddle's syndrome, an inheritable form of human hypertension. Mapping of these mutations on the ENaC gene indicated that inactivation of PY (proline rich) motifs is responsible. These observations lead to the hypothesis that the channel interacts, via its PY motifs, with the WW domains of ubiquitin-protein ligases of the Nedd4/Nedd4-like family. It is now well established that the cell surface expression of ENaC is controlled via ubiquitylation by members of this protein family and that ubiquitylation is regulated by the aldosterone-induced protein serum and glucocorticoid induced kinase 1 (SGK1) [14]. The relationship of Renin-Angiotensin-Aldosterone-System-BP-Regulation via ENaC and the involvement of several other gene products will be decribed in the following chapters.

2.1. The Renin-Angiotensin Aldosterone System (RAAS)

The RAAS plays an important role in regulating blood volume, arterial pressure, cardiac and vascular functions. The importance of this system is best illustrated by considering the response to lowered blood pressure (Figure 9). When the rapid nervous response, acting via vasoconstriction, is insufficient to bring the blood pressure back to the normal values, then hormonal control of the total body fluids must also occur. Renin, generated from prorenin, is released from cells of the juxtaglomerular apparatus of the kidneys, and circulates throughout the body. Renin secretion is inhibited by increases, and stimulated by decreases in intracellular calcium (Ca^{2+}) concentration. Voltage-sensitive Ca^{2+} channels in the plasma membrane are activated by membrane stretch, which correlates with arterial pressure and is assumed to mediate inhibition of renin secretion. Renin secretion is also stimulated when the concentration of Na^+ and Cl^- at the macula densa decreases [15, 16] or due to sympathetic discharge.

Renin catalyses the proteolytic cleavage of angiotensinogen, which is constitutively, secreted by the liver, forming the decapeptide angiotensin I (AI). Eventually, angiotensin I reaches the lungs where angiotensin converting enzyme (ACE) cleaves two amino acids to form the active octapeptide, angiotensin II (AII).

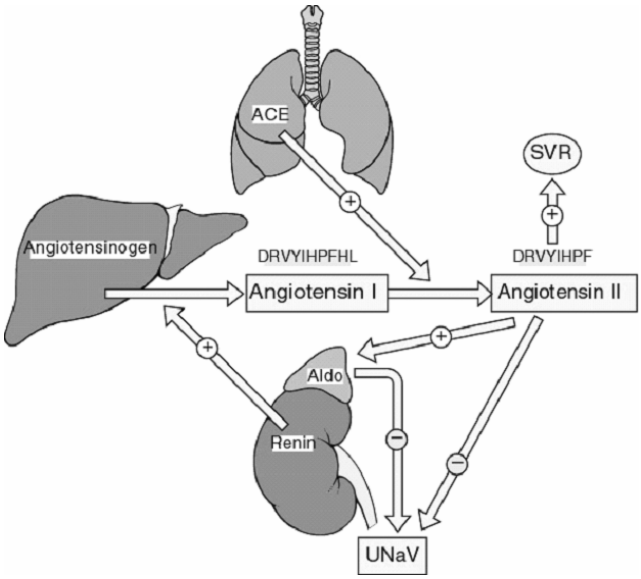


Figure 9. Schematic representation of the positive and the negative feedback loops of the renin-angiotensin-aldosterone system (RAAS).
SVR = systemic vascular resistance
Adapted from www.kidneyatlas.org.

Angiotensin II carries out various functions: First, it induces vasoconstriction that leads to an increase in blood pressure and, subsequently, to an increase in renal blood flow and glomerular filtration rate (GRF). Second, it activates the biosynthesis of the mineralocorticosteroid hormone aldosterone in the adrenal gland (Figure 9). The resulting increase in aldosterone level is then responsible for the stimulation of the Na^+ reabsorbing machinery in aldosterone-responsive cells.

The principle of aldosterone action is the retention of sodium. Where sodium goes, so follows the associated ions and water. Therefore, aldosterone profoundly affects fluid balance by affecting extracellular fluid volume. Aldosterone acts on the responsive segment specific cells localized in the distal nephron causing an increase in sodium reabsorption (see next paragraph). As a result, the tissue concentration of Na^+ in this area increases. Water reabsorption from the collecting duct depends on the surface expression of the ADH-sensitive AQP2 and is driven by simple osmosis due to high Na^+ and urea concentration of the surrounding tissue. The resulting increase in the body water also affects the blood pressure increasing it to normal level.

2.2. Aldosterone, Aldosterone Sensitive Distal Nephron (ASDN), Mineralocorticoid Receptor (MR) and Transepithelial Na^+ Transport

Aldosterone is a steroid hormone and belongs to the subgroup of mineralocorticoid hormones. It is secreted from the adrenal cortex after stimulation by the renin-angiotensin system (RAAS), kalaemia and, to some extent, ACTH. As for other steroid hormones, it binds to a cytosolic receptor and the complex then translocates to the nucleus where it increases or represses the expression of target genes via binding to hormone response elements (HRE) (i.e. SGK1). These receptors are members of the family of ligand-activated nuclear hormone receptors.

Two types of steroid receptors that bind aldosterone have been identified a type I receptor (mineralocorticoid receptor; MR), which is restricted to aldosterone responsive tissues and a type II receptor (glucocorticoid receptor; GR) [17-19]. Aldosterone binds with a high affinity (Kd ~1 nM) to the MR and with a low affinity to the GR (Kd ~50 nM). The enzyme 11-β-hydroxysteroid dehydrogenase type 2 (11-β-HSD-2) modifies glucocorticoids that also exhibit a high affinity for the MR, in the aldosterone target cells, thus providing a mechanism to protect the MR from glucocorticoid action (Figure 10.). 11-β-HSD-2 transforms cortisol (dominant glucocorticoid in humans; corticosterone: dominant glucocorticoid in rodents) into its corresponding cortisone [20], which has a much lower affinity for the MR. At high concentrations (10^{-7}-10^{-6} M) aldosterone binds to the GR and exerts specific actions. In A6 cells as well as mpkCCD_{c14} cells for example, most of the aldosterone effect is exerted via the GR.

The nephron portion that responds to aldosterone is referred to as ASDN, standing for ***A***ldosterone ***S***ensitive ***D***istal ***N***ephron. Morphologically, it contains the second half of the distal convoluted tubule (DCT2), the connecting tubule (CNT), the cortical collecting duct (CCD) and the medullary collecting duct (MCD). The aldosterone-responsive cells, or the segment-specific cells, are functionally defined as those expressing ENaC, the mineralocorticoid receptor (MR) and the enzyme 11β-HSD2. These segments are the site where the fine-tuning of the final urinary Na^+ excretion takes place. The Na^+ reabsorbing machinery is formed by apically located ENaC, which mediates Na^+ influx, and by the Na^+,K^+-ATPase, that extrudes Na^+ across the basolateral membrane to the blood side.

Aldosterone controls Na^+ reabsorption by regulating both the short- and long-term expression of both transport proteins. Additionally both transporters are regulated by a complex network of local factors (Figure 11.).

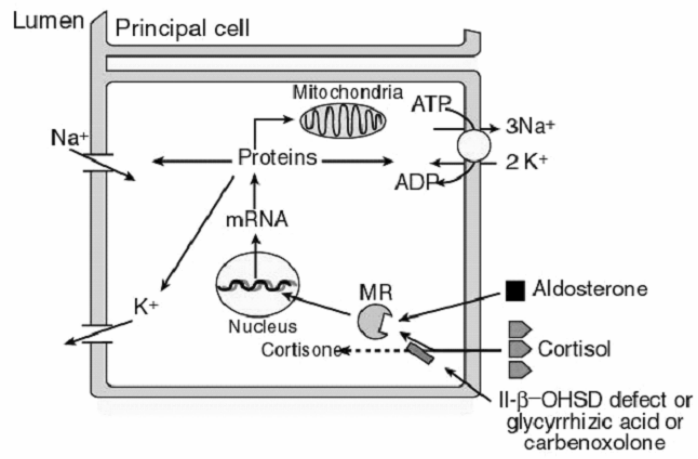


Figure 10. Simplified scheme of the principal cell (segment specific cell) of the ASDN with the epithelial sodium channel (ENaC), the Na^+,K^+-ATPase, the mineralo-cortiocid receptor (MR) and 11β-HSDS2
Adapted from www.kidneyatlas.org.

The electrochemical gradient, i.e. the driving force for sodium influx, is maintained by the activity of the basolaterally located Na^+,K^+-ATPase and the K^+ conductance of both membranes.

Functional and morphological studies [21, 22] clearly indicated that in rats and probably also in humans, the ASDN is composed of the late portion of the distal convoluted tubule (DCT2), in which ENaC is co-localized with the DCT-specific thiazide-sensitive sodium chloride co-transporter, the connecting tubule (CNT), as well as the cortical and the medullary part of the collecting duct, respectively. All along the ASDN, intercalated cells are interspersed between the sodium transporting segment-specific cells, termed DCT cells, CNT cells and the collecting duct cells (also called principal cells). In contrast to the segment-specific cells, the intercalated cells show different characteristics and play a role in proton and bicarbonate secretion and/or reabsorption.

Aldosterone has the opposite effect on serum levels of potassium than of sodium since to a large extent potassium is excreted in exchange for sodium that is reabsorbed via ENaC. Salivary and sweat glands as well as the distal colon respond to aldosterone to prevent excessive sodium loss. Aldosterone levels can increase fluid retention in diseases such as congestive heart failure and liver cirrhosis. Certain diuretics act by antagonizing aldosterone. In contrast to most diuretics which cause potassium loss, the aldosterone antagonists, i.e. K^+ sparing diuretics, increase blood potassium and are sometimes used to accomplish this effect.

2.3. The Early and Late Effects of Aldosterone in the ASDN

The best known action of aldosterone in ASDN cells is to increase the ENaC mediated sodium conductance at the apical membrane and the activity of the Na^+,K^+-ATPase pump in the basolateral membrane. The action of aldosterone has been operationally divided into three physiologically more or less distinct phases, which are:

1) a latent period of less than 30 minutes, where the initial changes in gene transcription and protein synthesis starts;
2) an "*early response*" period, with a 2- to 3- fold increase in sodium reabsorption within 3 hours of aldosterone action mainly due to the increased activity of pre-existing ENaC and Na^+,K^+-ATPase [23, 24];
3) a "*late response*" period of up to 24 hours with not only a further increase in the activity of ENaC but also that of the Na^+ pumps.

However, this division is schematic and does not appear to be appropriate for the *in vivo* situation in mammals. First, this operational division is only meaningful in extreme conditions such as those imposed in experimental situations (all or none situation). Second, ENaC subunit upregulation that occurs in the early phase (see below) is an 'anabolic type' effect. Finally, the concept of anabolic response is also applicable to the induction of regulatory proteins, because these proteins represent constitutional elements of regulatory cascades.

Undeniably, aldosterone induces compared to a substantial increase of α-ENaC protein abundance a modest increase in the α-ENaC subunit mRNA expression but does not affect the mRNA expression of the β- and γ-ENaC subunit in the rat and mouse CCD [25-27]. The rapid effect of aldosterone on ENaC is probably due to the observed increase in the insertion of channels in the apical membrane [25, 27, 28].

The connection between the activated hormone/receptor complex and the ultimately higher ENaC conductance has remained obscure until the identification of SGK1 (Figure 11.).

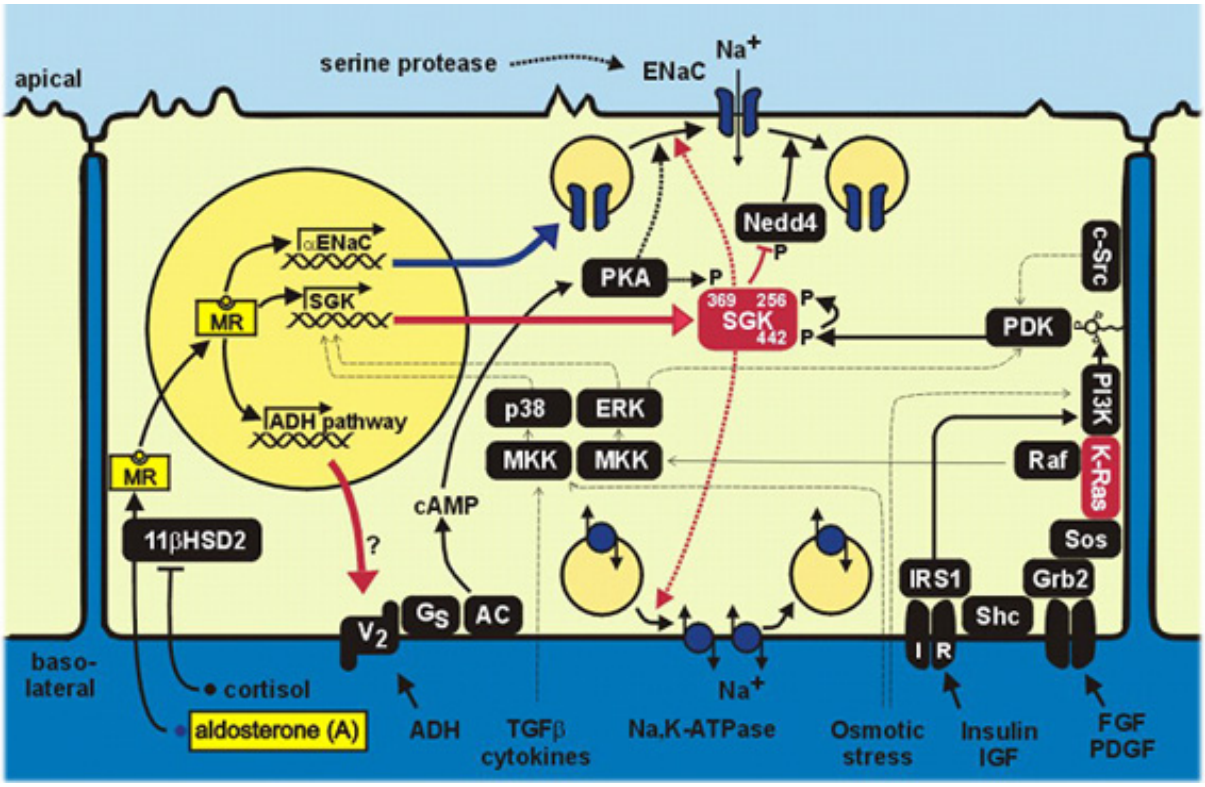


Figure 11. Schematic representation of regulatory pathways participating or modulating the aldosterone action in segment-specific cell of the aldosterone-sensitive distal nephron The thick arrows indicate transcriptionally mediated inductions by aldosterone of gene products which impact on sodium reabsorption via the epithelial sodium channel (ENaC). Medium arrows indicate regulatory pathways, the importance of which are established in the context of sodium reabsorption. Medium arrows are stippled when the precise site of impact or the existence of intermediate steps is not established. Thin, stippled arrows indicate pathways that have been described in other cell types and might modulate aldosterone action in the aldosterone-sensitive distal nephron (ASDN). AC, Adenylyl cyclase; ADH, anti-diuretic hormone (vasopressin); cAMP, cyclic adenosine 3',5'-monophosphate; ERK, mitogen-activated protein kinase (MAPK) of ERK (extracellular signal-regulated kinase) type; FGF, fibroblast growth factor; G_S, trimeric G-protein α_S subunit; 11βHSD2, 11-β-hydroxysteroid dehydrogenase type 2; IGF, insulin-like growth factor; IRS1, insulin receptor substrate 1; MKK, MAPK kinase; MR, mineralocorticoid receptor; p38, MAPK p38; PDGF, platelet-derived growth factor; PDK, phosphoinositide-dependent kinase; PI3-K, phosphoinositide 3-kinase; PKA, protein kinase A; TGFβ, transforming growth factor; V2, vasopressin receptor type 2. Note the regulatory pathway that links the transcriptional regulation of SGK1 to the cell-surface expression of ENaC via Nedd4-2.
Adapted from Loffiing et al. [326].

The coexpression of SGK1 with the three ENaC subunits in the *Xenopus laevis* oocytes produces much higher amiloride inhibitable currents than occur in the absence of co-expression [29-32].

Other substances, such as hormones and intracellular factors like pH, calcium, regulatory proteins, can also affect ENaC conductance on several levels, i.e. via mRNA expression, protein synthesis and degradation, cellular distribution and (possibly) function. One interesting possible regulatory factor is the channel activating protease (CAP) [33].

Corticosteroids, such as aldosterone and dexamethasone, have been shown to have both short- and an long-term effects on the Na^+,K^+-ATPase. The long term increase in pump activity observed in several model systems is mediated by an increase in Na^+,K^+-ATPase pump expression [34-36]. The corticosteroid hormone/receptor complex interacts directly with regulatory elements of both the α1 [34] and β1 subunit genes [37]. Aldosterone has also short-term effects on pump activity. This effect can be mediated by an increase in the amount of Na^+ in the cell, via its entry through ENaC. Since it is a rate limiting substrate, intracellular Na^+ ($[Na^+]_i$) then stimulates the activity of the Na^+,K^+-ATPase [35, 38]. In addition, it might also prompt the translocation of the Na^+,K^+-ATPase pump to the plasma membrane [39]. On the other hand, aldosterone can also mediate the upregulation of Na^+,K^+-ATPase in A6 cells [40, 41], independent of Na^+ influx, in a transcription- and translation-sensitive manner.

K-Ras2 [42] might also play an important role in the increase in overall Na^+ reabsorption but the mechanism of action has not yet been shown. K-Ras possibly activates lipid kinases causing an increase in phosphatidyl-inositol-2 phosphate (PIP_2) [43] which has been shown to stimulate ENaC in excised membrane patches of A6 cells [44]. Another possibility is that the traditional Ras effector, Raf kinase, might stimulate MKK (MAPK kinase) which then acts as upstream regulator of SGK1. Furthermore, Ras can also activate PI3 kinase which acts on SGK1 activity via PDK1. Cell-specific differences may occur.

As depicted in Figure 11., several signaling pathways affect the function of ENaC via other receptors in response for instance to IGF1 [45], insulin, PDGF, ADH and FGF. Ultimately cross-talk among various signalling pathways will have to be considered in order to understand the overall context in which aldosterone exerts its action. Such approaches have already been attempted and will be considered in the discussion section of the thesis.

In the following chapters some key gene products, ENaC, Na^+,K^+-ATPase, SGK1 and Nedd4-2 of the scheme shown in Figure 11. will be described in detail.

2.3.1. The Epithelial Sodium Channel (ENaC)

ENaC is a member of the DEG/ENaC family of ion channels. Individual members of this family have diverse functions, which include mechanosensation, proprioception and salt regulation [46]. ENaC is an important regulator of Na^+ homeostasis and thus plays a major role in maintaining extracellular volume and blood pressure. It is expressed in the kidney distal nephron and in the renal collecting duct, where it regulates urinary Na^+ excretion, which is important for maintaining a balance of fluids, Na^+ and other electrolytes in the body. ENaC is responsible for active Na^+ reabsorption in epithelial tissues such as the renal segment-specific cells of the CNT/CCD, the distal colon (under the control of aldosterone), the lung, the parotid gland and the sweat- and salivary-ducts [47]. It is localized to the apical membrane of polarized epithelia and it shows axial heterogeneity along the ASDN. Its expression is most pronounced in the early ASDN portions (late DCT and early CNT) and expression progressively decreases in downstream segments (late CNT and CD) [26, 27]. The ENaC protein expressed in the distal colon and sweat- and salivary-ducts functions to reduce the amount of Na^+ loss [47]. In the lung, ENaC maintains the correct composition of the fluid that lines lung epithelia, especially during the transition from liquid-filled to air-filled neonatal lung [47, 48]. Na^+ transport through ENaC occurs by electro-diffusion rather than by coupling with transport of co-solutes or through an energy-dependent process. ENaC function can be blocked by amiloride, a K^+-sparing diuretic [47, 49]. Functional ENaC is generally composed of three homologous subunits, named α, β and γ. Similar to other DEG/ENaC family members, each ENaC subunit (Figure 12.) has a conserved protein structure, consisting of two hydrophobic trans-membrane regions (TM1, TM2), an large extracellular loop with highly conserved cysteine residues, and short intracellular amino and carboxyl termini [46, 50, 51].

The subunit stoichiometry of functional plasma membrane-associated ENaC is controversial. However studies have provided evidence that support a tetrameric, 2α:1β:1γ [52-54], or lately a trimeric [55] stoichiometry. The α subunit was first cloned from rat by two independent groups, who both identified a cDNA encoding a predicted molecular mass of 79 kDa protein [56, 57]; later the β and γ rat ENaC subunits were cloned by Canessa et al. [58]. It was found that injection of *Xenopus*

oocytes with all three subunits resulted in an amiloride-sensitive Na^+ current more than 100-fold larger than that of αENaC alone. Therefore, fully functional ENaC consists of all three subunits [58].

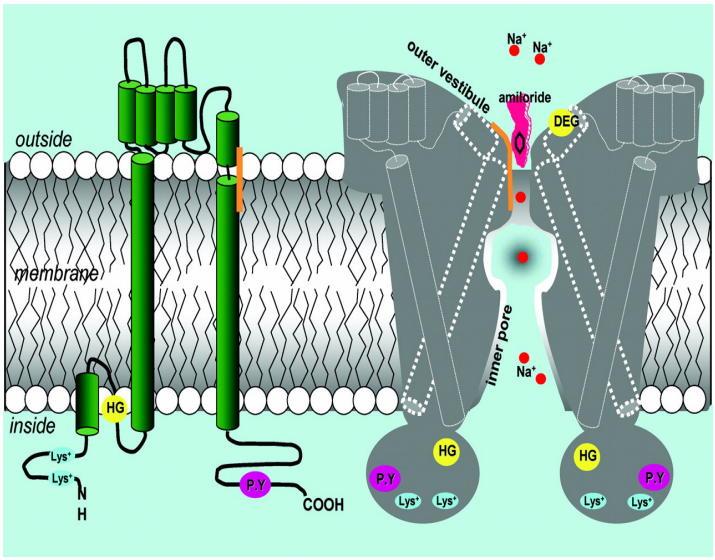


Figure 12. Schematic representation of the ENaC channel in the membrane. (Left) Secondary structure predictions and membrane topology of one ENaC subunit. (Right) ENaC channel model. Cylinders represent putative α helices. P, extracellular pore helix; HG, conserved His and Gly residues among all of the members of the ENaC/degenerins family members; P.Y, PY motif common to the αβγ-ENac subunits; DEG, degenerin site in the P-helix.
Adapted from Gramling Medical Illustration.

Proline-rich motifs are found in the C-terminus of the αβγENaC subunits [59], in particular a PPxY sequence (PY motif) that represents a characteristic pattern for WW peptide ligands, like Nedd4-2, an ubiquitin ligase, exhibits. These PPxY sequences in the β and γENaC subunits are the target sequence for mutations that cause Liddle's syndrome (pseudohypoaldosteronism) [60-62]. Nedd4-2 catalyzes the attachment of ubiquitin moieties on the channel for endocytosis and degradation. Mutations in the PY motif alter this process [63]. When ENaC gets ubiquitylated, multiple lysine residues in the N-termini of α (K47 and K50) and γ subunits (K6 to K13) are substrates for attachment of the ubiquitin moieties [64].

The importance of each individual ENaC subunit is supported by the corresponding mouse knockout phenotypes. α-, β- and γENaC knockout mice die shortly after birth from a failure to clear lung fluid (α ENaC, [65]), show increased urinary Na^+ but normal lung fluid clearance (βENaC, [66]) and have electrolyte disturbances and a minor lung fluid clearance defect (γENaC, [67]).

2.3.1.1. ENaC Regulation

ENaC activity must be tightly regulated since it is the rate-limiting step in Na^+ absorption in tissues such as the kidney. ENaC is located on the apical membrane allowing the entry of Na^+ into the cell, which then exits the cell through the basolaterally located Na^+/K^+ ATPase [68].

Typically ENaC shows a unitary conductance of 4-5 pS for Na^+ [58, 69, 70] and of 9-10 pS for Li^+ [58, 69]. It also shows a strong selectivity of Na^+ over K^+.

ENaC regulation occurs by changing the channel open probability (P_o) and/or altering the number of channels at the plasma membrane. The latter appears to be more important in controlling Na^+ absorption. A number of hormonal and non-hormonal mechanisms regulate ENaC density at the plasma membrane. Hormones which regulate ENaC include aldosterone, insulin and vasopressin [47]. Non-hormonal regulation of ENaC can occur in response to changes in ion concentrations, by the cystic fibrosis transmembrane conductance regulator (CFTR) and by proteases [47, 48].

In the kidney, ENaC is highly regulated by various hormonal pathways, such as those linked to aldosterone, vasopressin, and insulin [71]. Although in recent years, considerable progress has been made in understanding the regulatory action of hormones many of the molecular mechanisms involved remain unsolved. Genetic evidence supports the current view that ENaC expression and activity is regulated via ubiquitylation and phosphorylation [14].

2.3.1.2. Hormonal Regulation of ENaC

The molecular mechanisms by which hormones regulate ENaC activity can be classified as: (*1*) change of the number of channel molecules expressed at the plasma membrane, (*2*) change of the open probability (P_o) of the channel, and (*3*) change of the unitary conductance of the channel [72]. The most likely candidate proteins that could up-regulate ENaC activity by their increased synthesis are the ENaC subunits themselves, but this is considered to be a late response that is initiated 6-24 hours following aldosterone stimulation [47, 73]. Furthermore, increased ENaC subunit synthesis does not include all subunits; rather the effect is tissue-specific with increased αENaC in the kidney and increased β and γENaC

mainly observed in the distal colon [74-76].

An effort to identify in Xenopus laevis A6 cells genes that are up-regulated during the early response to aldosterone stimulation, [42]) identified the small G protein KRas2A. It was subsequently shown that K-Ras2A over-expression in *Xenopus* kidney cells (A6) significantly increased the inward movement of Na^+ [77]. A direct role for K-Ras2A-mediated Na^+ channel activation remains to be determined [78]. Another study has shown that aldosterone can stimulate carboxymethylation of βENaC and this may increase ENaC activity [79].

Another possible avenue for ENaC stimulation by aldosterone is via the signaling molecule phosphoinositide 3-kinase (PI3-K). It has been shown that PI3-K is required for aldosterone-stimulated sodium reabsorption. ENaC stimulation may occur via the activity of the PI3K phosphorylation targets; phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) [80]. Recent evidence suggests that PtdIns(3,4)P2 and PtdIns(3,4,5)P3 affect ENaC activity by increasing channel P_o [81]. It has also been shown that the PI3-K signalling pathway activates SGK1 by phosphorylation [82, 83]. From this it would appear that aldosterone potentially activates SGK1 by increasing transcription and by the PI3-K pathway-mediated phosphorylation of SGK1 [78].

The insulin-like growth factor 1 (IGF-1) [45, 84] and the peptide hormone insulin [47] are known to activate Na^+ channels in a variety of epithelial cells. They function by binding to the IGF-1-receptor or insulin receptor respectively, which triggers intracellular signalling. Studies have provided evidence of insulin-stimulated Na^+ channel activation being mediated by protein kinase C (PKC) [85] and PI3-K [86], and IGF-1 dependent Na^+ channel activation by PI3-K [45]. A role for kinase-mediated ENaC stimulation in response to insulin is further bolstered by the finding that insulin stimulation leads to β and γENaC phosphorylation [87]. Insulin stimulation has been proposed to affect ENaC activity by increasing the P_o [88] and by increasing ENaC density at the plasma membrane [89, 90]. The finding that the PI3-K pathway is stimulated by aldosterone, insulin and IGF-1 has led to suggestion that ENaC stimulation by those hormones may be carried out by SGK1 [91].

Vasopressin is a peptide hormone that increases Na^+ channel activity as rapidly as 5 minutes following stimulation. Following binding to its V2 receptor, adenylate cyclase is activated, which results in the production of the second messenger cyclic AMP (cAMP). cAMP is believed to activate kinases that are able to up-regulate ENaC activity by an unknown mechanism. It has been suggested that this can occur by increasing the channel density at the plasma membrane and by increasing the P_o [47].

2.3.1.3. Non-hormonal Regulation of ENaC

Na^+ feedback inhibition is a well-characterised mechanism of ENaC down-regulation. In addition to intracellular Na^+, Ca^{2+}, pH, anions and extracellular Na^+ have also been implicated in the control of Na^+ channel activity [47, 92]. Strong evidence for an inhibitory action of increased intracellular Na^+ on Na^+ channels was reported by Komwatana et al. [93].

Nedd4 has been found to down-regulate ENaC via its specific interaction with binding sites on channel subunits which are mutated in Liddle's syndrome [63, 94]. A detailed presentation of this pathway will be given in later chapters.

CFTR secretes Cl^- in response to cAMP and is known to affect ENaC activity. It was shown by Mall et al. [95] that CFTR down-regulates ENaC in normal human lung epithelia, but not in the lung epithelia of cystic fibrosis patients that have mutant CFTR. It has been suggested that CFTR and ENaC physically interact thereby eliciting regulatory effects [96]. It has also been proposed that various CFTR accessory proteins might facilitate regulatory interactions with ENaC [97].

To be fully active, ENaC requires considerable post-translational modifications, including proteolytic cleavage. It has been shown that in ENaC-expressing epithelia only a very small proportion of ENaC is expressed on the plasma membrane, with a majority of the channel subunits residing in pre-golgi compartments as immature forms where they await degradation [98]. Subunits are glycosylated at a number of sites in the extracellular loop, but during translocation to the plasma membrane these modifications undergo complicated processing [99].

Further, it was shown that proteolytic cleavage of the α and γ subunits is another important step in the maturation of ENaC. The cleavage sites are located in the extracellular loop, near the amino-membrane-spanning domain for each subunit, with the resulting fragments remaining associated with the channel [100]. It was subsequently found that the proprotein convertase, furin, is responsible for cleavage of the α and γ subunits, which up-regulates ENaC activity [101].

Another protease, channel-activating protease 1 (CAP1), has been shown to increase ENaC activity [33]; however, it is not known whether it functions by cleaving ENaC or another regulatory protein. There is also evidence that CAP1 (prostasin) is induced by aldosterone [102].

2.3.2. The Na^+,K^+-ATPase

The basic function of Na^+,K^+-ATPase is to maintain the high Na^+ and K^+ gradients across the plasma membranes of most higher eukaryotic cells. For its activity the pump uses the energy from the hydrolysis of the phosphate bond in the ATP. The ion gradient that results from its function plays an important role as the driving force for a variety of Na^+ dependent secondary transporters, like those of glucose and amino acids, and enables the K^+ conductance to stabilize the membrane potential close to the K^+ Nernst potential.

The minimal functional unit of the Na^+,K^+-ATPase is an oligomer composed of stoichiometric amounts of either of the α (α1, α2, α3 or α4) and either of the β (β1, β2 and β3) subunit isoforms. Whereas the α subunit possesses 10 predicted transmembrane domains and a mass of 112 kDa, the β subunit polypeptide is a type II membrane protein with a single transmembrane domain that exhibits a molecular weight between 40 and 60 kDa. The two subunits differ in their roles, the α-subunit being the actual catalytic subunit. The additional γ subunit has a molecular weight of only 8-14 kDa and belongs to a family of small membrane proteins that appear to modulate the passage of ions across the plasma membrane. The γ subunit is not required for normal Na^+,K^+-ATPase activity [103] but nevertheless it seems to modify the sodium/potassium affinity of the enzyme.

The hypothesis, that SGK1 is also involved in Na^+,K^+-ATPase regulation was confirmed in Xenopus laevis oocytes, since it was demonstrated, that SGK1

increases the activity of the Na^+,K^+-ATPase by increasing its cell surface expression [104, 105].

CHIF, a small protein related to the γ subunit, has been shown to localize to the basolateral membrane of segment-specific cells of the ASDN. It represents an example of the differential aldosterone action in kidney ASDN versus the distal colon. In contrast to the colon, CHIF is not regulated by aldosterone in the kidney ASDN but rather by a low sodium diet. Its role in the context of aldosterone action remains unclear, but indirect evidence points to a regulatory function in epithelial potassium transport [106].

2.3.3. The Serum- and Glucocorticoid induced Kinase (SGK1)

SGK1 was originally identified as a kinase that is inducible by serum and glucocorticoids and belongs to the early aldosterone-induced gene products in kidney (ASDN) [14]. It is a member of the AGC (cAMP dependent, cGMP dependent, and protein kinase C) family of Ser/Thr kinases and shares approximately 50% identity in its catalytic domain with other AGC kinases such as protein kinase A, Akt, or protein kinase C [107]. There are two close homologues, SGK2 and SGK3 (or CISK), that share approximately 80% sequence identity with SGK1 [108, 109]. They also share a high degree of similarity with Akt/protein kinase B. SGK1 was one of the first early aldosterone-induced gene products detected in renal epithelial cells [26, 29, 31, 110-113].

Interest in SGK1 was considerable because it stimulates ENaC activity in oocytes [26, 29, 31, 91, 110, 114-120]. This increase in ENaC activity was shown to be mostly due to elevated channel cell surface expression [26, 115, 116, 119], although one report also observed an effect on P_o [118]. Further evidence for a role in epithelial Na^+ transport came from transduction experiments in renal epithelial cells, which demonstrated that expression of SGK1 in various cell lines increased transepithelial Na^+ currents [121-124].

Alvarez De La Rosa et al. [123] observed a 4-fold effect on ENaC cell surface expression as well as an increase of 43% in P_o in A6 cells that were transduced with constitutive active SGK1. It is not clear how SGK1 stimulates the P_o of ENaC, as the SGK1-dependent phosphorylation site on αENaC, described by Diakov et al. [125] below, is not conserved in *Xenopus laevis* αENaC. SGK1 activity is

known to be regulated by the phosphatidylinositol-3 kinase pathway and needs to be phosphorylated by PDK1, demonstrating that its function depends on the activity of this pathway. Indeed, evidence was provided by several groups that stimulation of Na^+ transport by aldosterone and/or SGK1 is inhibited by phosphatidylinositol-3 kinase inhibitors [80, 91, 126]. Antisense [124], RNA interference [113], or overexpression of catalytically inactive SGK1 [121, 122, 124] further corroborates the role of SGK1 in positive regulation of transepithelial Na^+ transport. The strongest evidence for such a function comes from the SGK1 knockout mice, which present a pseudohypoaldosteronism type 1 phenotype when kept under low-Na^+ diet [127].

2.3.3.1. Tissue distribution of SGK1

SGK1 expression is induced by a very large spectrum of stimuli in addition to glucocorticoids and serum [128]. These include aldosterone [29, 110], TGF-β [120, 129-131], vitamin D_3 [132], glucose [131], endothelin-1 [131], granulocyte-macrophage colony–stimulating factor (GM-CSF) [133], fibroblast growth factor (FGF) [134], platelet-derived growth factor (PDGF) [134], p53 [135-138] and many others. Sgk1 [107, 108, 139] is broadly distributed, with expression in many tissues, including the brain, placenta, lung, liver, pancreas, kidney, heart, and skeletal muscle. In situ hybridization studies localized Sgk1 mRNA in several epithelial and/or nonepithelial cells within the brain [140-144], eye [145, 146], lung [147], liver [130], ovary [148], pancreas [149], intestine [129], and kidney [29, 150, 151].

The aldosterone-dependent induction of Sgk1 occurs specifically in the cytoplasm of ENaC-positive cells of the ASDN, whereas Sgk1 expression in other nephron portions such as the thick ascending limb or the proximal tubule is not increased by aldosterone [27]. The subcellular localization is consistent with the reported intracellular localization of Sgk1 in stably transfected CCD cells [152], in mammary tumor cells expressing exogenous or endogenous Sgk1 [153], and in *C. elegans* expressing GFP-tagged SGK1 [154].

It is important to note that the subcellular distribution of Sgk1 may change according to the stimulus received. Sgk1 can be localized to the nucleus, to the cytoplasm and/or at the plasma membrane [148, 153, 155-157].

2.3.3.2. Mechanisms of SGK1 Action

Several mechanisms have been proposed for SGK1-dependent regulation of ENaC. Wang et al. [91] showed that SGK1 directly interacts with the C-terminus of βENaC. Diakov et al. [125] identified a consensus phosphorylation site on the C-terminus of αENaC (S621) and found by the outside-out patch-clamp technique and inclusion of recombinant SGK1 in the pipette that this site may be important for ENaC stimulation. However, mutation of this site to alanine (S321A) does not impair the stimulatory effect of SGK1 (as well as of SGK2 and SGK3) on amiloride-sensitive Na^+ currents in *Xenopus laevis* oocytes [158]. Demonstration that αENaC indeed becomes phosphorylated by SGK1 will be required to shed some light on this mechanism.

An alternative model proposes that Nedd4-2 is a substrate of SGK1, as Nedd4-2 contains three consensus phosphorylation sites for SGK1 [114, 115], and SGK1 itself contains a PY motif, rendering it possible that SGK1 and Nedd4-2 interact *via* a PY motif/WW domain (Figure 13.).

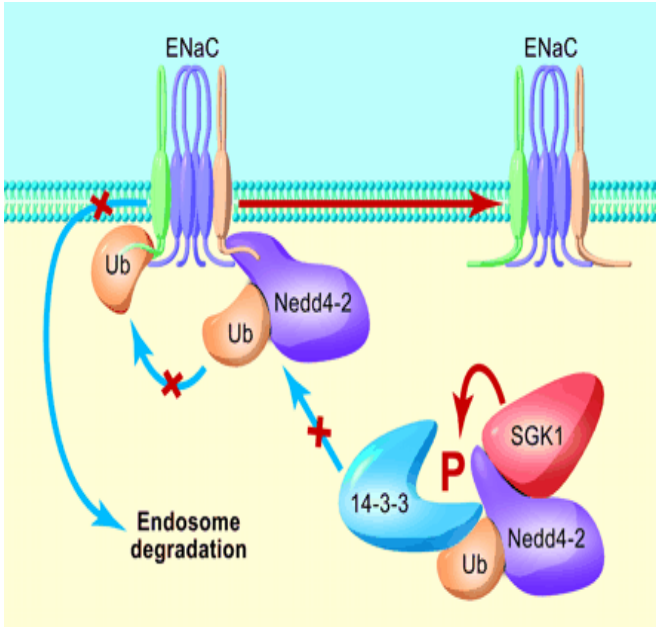


Figure 13. Model of regulation of the ENaC by Sgk1 and Nedd4-2. Induction of aldosterone leads to the expression of Sgk1. Sgk1 interacts via PY motif/WW domains with Nedd4-2 and phosphorylates Nedd4-2 on Ser444. Phosphorylated Ser444 serves as binding site for 14-3-3, which hinders interaction of Nedd4-2 with ENaC. This interferes with ENaC ubiquitylation, internalization, and degradation in the lysosomal pathways, consequently leading to accumulation of ENaC at the cell surface. Adapted from Gramling Medical Illustration (Staub and Verrey).

In support, it was demonstrated that SGK1 does interact with Nedd4-2 [114, 159] and that the PY motif on SGK1 is partly required for SGK1-dependent stimulation of ENaC in *Xenopus laevis* oocytes [115]. In oocytes, it was also shown that SGK1 induces phosphorylation of Nedd4-2 on two sites, primarily on Ser444 but also on Ser338. Moreover, mutation of these sites interfered with SGK1-dependent phosphorylation of Nedd4-2 and stimulation of ENaC [115].

It was further demonstrated that SGK1 reduced the interaction between Nedd4-2 and ENaC, indicating that SGK1 impairs ENaC ubiquitylation and subsequent internalization.

Taken together, these data suggest that one of the mechanisms by which aldosterone regulates ENaC function is by transcriptionally inducing SGK1 that in turn phosphorylates Nedd4-2. As expected from such a mechanism, the phosphorylation of Nedd4-2 on Ser444 is increased upon aldosterone treatment in mpkCCD$_{c14}$ cells and in adrenalectomized rats [126]. When phosphorylated, Nedd4-2–Ser444 is part of a consensus binding site for 14-3-3 proteins, suggesting that it is the binding of 14-3-3 proteins to Nedd4-2 that sterically interferes with the interaction of Nedd4-2 with ENaC and prevents ENaC ubiquitylation [117]. Bhalla et al. (2006) [160] showed that 14-3-3 proteins bind to phosphorylated Nedd4-2 and interfere with Nedd4-2/ENaC interaction and ENaC ubiquitylation.

3. Ubiquitylation

Ubiquitin is a highly conserved 76 amino acid protein expressed in all eukaryotes. Ubiquitin is covalently linked to protein substrates in a tightly regulated form of post-translational modification known as ubiquitination, or ubiquitylation. This can occur as: mono-ubiquitylation, the attachment of a single ubiquitin molecule to a position within the substrate; poly-ubiquitylation/multi-monoubiquitylation, attachment of a chain of ubiquitin molecules to the substrate; or combinations of the two [161, 162]. The most well characterised consequence of ubiquitylation is substrate degradation, which occurs by either the proteasomal or lysosomal pathways [163]. A diverse array of proteins is down-regulated via ubiquitylation, such as cell cycle regulators, transcription factors, tumour suppressors, receptors and ion channels [162-165]. Another non-degradatory role of ubiquitylation appears to be in facilitating protein trafficking, which has emerged from the discovery of domains that undergo protein-protein interactions with ubiquitylated ligands [166].

Since the discovery of ubiquitin, a family of ubiquitin-like protein modifiers (UBLs) has been identified, which includes SUMO/Sentrin, Nedd8/Rub1, ISG15/UCRP and FAT10 [167, 168].

Ubiquitylation is a complex process involving three major classes of enzyme. Ubiquitin is first activated by an ubiquitin-activating enzyme (E1) in an ATP-dependent manner, which results in the formation of a thioester bond between the ubiquitin carboxyl terminus and an internal cysteine of the E1 [169]. Ubiquitin is then transferred to the active cysteine of an ubiquitin-conjugating enzyme (E2) [165]. Following this, the E2-ubiquitin conjugate interacts with an ubiquitin-protein ligase (E3), which results in formation of an isopeptide linkage between the carboxyl terminal glycine of ubiquitin and, most commonly, a lysine residue within the substrate protein [164]

Hundreds of E3 enzymes participate in substrate recognition [14]. These enzymes can be divided into two major protein families, namely the RING finger [170] and the HECT (homologous to E6-AP carboxyterminal) domain-containing proteins. Nedd4 belongs to the family of HECT domain ubiquitin-protein ligases

[171].

Poly-ubiquitylation results from the sequential attachment of ubiquitin molecules to lysine residues within ubiquitin molecules already attached to a substrate [165]. Of the seven lysine residues within ubiquitin, poly-ubiquitin chains are mostly linked through K48, but can also occur through K63, K29 or K11 [161, 172]. Poly-ubiquitin chain formation through K48 is normally a signal for protein degradation by the proteasome, while chains formed through K63 are known to be involved in nondegradation functions [161, 172]. Besides involvement in protein trafficking, DNA repair and histone modification, mono-ubiquitylation and multiple mono-ubiquitylation are strongly implicated in endocytosis and lysosomal degradation [162, 173].

3.1. The Proteasomal and Lysosomal Degradation

The proteasome is a large multi-catalytic complex responsible for the degradation of a majority of ubiquitylated soluble cellular proteins [174]. Typically, the proteasome degrades proteins containing poly-ubiquitin chains linked through K48, into short peptides [164]; however, certain non-ubiquitylated proteins can also be degraded by the proteasome [175]. The full-sized 26S proteasome is composed of three major complexes: a 20S cylinder-shaped catalytic core, with a 19S regulatory complex at either ends [174].

Lysosomes are intracellular organelles that contain acidic proteases. The protein contents of late endocytosis vesicles can be delivered to lysosomes, resulting in their degradation [176]. The number of receptors, channels and transporters at the plasma membrane can be controlled via endocytosis and lysosomal degradation in response to changing cellular environments. Following ubiquitylation of such proteins, their endocytosis occurs via invagination of the local plasma membrane that eventually forms an intracellular membrane bound vesicle. Endocytosed membrane proteins may be simply recycled back to the plasma membrane or directed to the lysosomal degradation pathway. Plasma membrane proteins that are degraded in this way include the platelet-derived growth factor β, epidermal growth factor and growth hormone receptors, and ENaC [177].

A common trigger for endocytosis of plasma membrane proteins is mono-ubiquitylation at an intracellular site, although in some cases a short ubiquitin chain linked through K63 is used [162]. Internalisation and lysosomal delivery are believed to be mediated by the action of ubiquitin-receptor proteins that recognise ubiquitylated proteins via ubiquitin-interacting protein domains [166, 173, 178]. In a similar way, ubiquitylation can also facilitate transport of newly synthesised proteins from the trans-golgi network to the lysosome for degradation [166, 178].

3.2. The Nedd4-family

Nedd4 is a HECT domain ubiquitin-protein ligase [14]. These proteins are characterized by a C-terminal highly conserved 40-kD enzymatic HECT domain, and a variable, N-terminal region, which is involved in substrate recognition. The HECT domain contains a cysteine, which is essential for catalytic activity [171]. Nedd4 is the founding member of a subfamily of HECT enzymes, which contains nine members [179]. These enzymes are composed of an N-terminal (Ca^{2+}-dependent phospholipid binding) C_2 domain [180], two to four WW domains [181], and the C-terminal HECT domain. They are involved in several different cellular functions [179, 182, 183]. The isolation of the WW domain containing Nedd4–1 suggested that ENaC may interact with members of this family via its PY motifs. Upon interaction, Nedd4/Nedd4-like proteins may ubiquitylate one or several ENaC subunits, in analogy to many plasma membrane receptors [184], and cause their internalization and targeting to the endosomal multivesicular body pathway and ultimately degradation in the lysosome.

Nedd4 was originally identified as a developmentally down-regulated mouse gene highly expressed in early embryonic central nervous system (CNS) [185]. It was subsequently found to be expressed widely in other embryonic and adult tissues [63, 186]. Other proteins were discovered in both simple and complex eukaryotes that share this domain architecture, which led to their classification as Nedd4-family member, or Nedd4-like, E3 proteins [183].

3.2.1. The C_2 Domain

The C2 domain was first identified in protein kinase C and can function as a Ca^{2+}-dependent phospholipid binding domain [187-189]. It consists of approximately 130 amino acids and is found in many proteins that function in signaling and membrane trafficking. A well studied C_2 domain-containing protein is synaptotagmin I, which is found in synaptic vesicles and is required for Ca^{2+}-dependent neurotransmitter release. The C_2 domain is a compact β-sandwich structure consisting of two four-stranded β-sheets, with three joining-loops at one end of the structure that bind Ca^{2+} [189]. The C_2 domain of Nedd4 has been shown to mediate localisation to the plasma membrane in response to Ca^{2+} [190]. There are examples of C_2 domains engaging in protein-protein interactions, such as the C_2 domain of the Ras-specific GTPase activating protein, P120GAP, which has been shown to interact with a number of proteins [191]. The C_2 domain of Nedd4 has also been shown to interact with the adaptor protein Grb10, in a Ca^{2+}-independent manner [192]. It has recently been reported that the C_2 domain of Rsp5, the sole Nedd4-family protein in *S. cerevisiae*, is required for localisation to endosomal membranes [193]. The C_2 domains of Nedd4-family proteins may be required to bring them in close proximity to substrates via associations with cellular phospholipids and by direct protein-protein interactions.

3.2.2. The WW Domains

The WW domain is a 35-40 amino acid protein-protein interaction module that was first identified as a repeat sequence in the YES-kinase-associated protein [194]. It was also identified by two independent groups, who named it the WWP [195] and Rsp5 domain [196]. In most cases, it contains two conserved tryptophan residues, hence the term WW domain [194], and conserved proline residue. Its structure consists of a three-stranded anti-parallel curved β-sheet, with the concave side forming a hydrophobic ligand-binding pocket [197]. WW domains are found in a large variety of proteins, and often in multiple copies. Some WW domain-containing proteins are implicated in diseases, including Alzheimer's disease, muscular dystrophy and Liddle's syndrome [198]. The PPxY sequence, known as the PY motif, was first identified in binding targets of the YES-kinase-

associated protein [199] and constitutes the most common WW domain-binding motif, including those recognised by Nedd4-family protein WW domains. There have been reports of variations to the basic PY motif, in particular replacement of the first proline residue by leucine (LPxY) [200-202]. Evidence that Nedd4-family protein WW domains can interact with various phosphorylated ligands in addition to the PY motif greatly expands the number of potential binding targets.

3.2.3. The HECT Domain

The HECT domain was first identified in E6- AP. This led to the identification of other HECT-containing proteins including Nedd4 and Rsp5. The HECT domains of these proteins each contained a conserved cysteine residue responsible for thioester formation with ubiquitin [171]. It was subsequently found that the amino region of the Nedd4 HECT domain could mediate interactions with the E2 UbcH5b [203]. Nedd4 has also been shown to receive ubiquitin from other E2s, including UbcH5a, UbcH5c, UbcH6 and UbcH7 [203, 204]. Therefore, it can be expected that the HECT domain of Nedd4-family proteins is critical for substrate ubiquitylation, which can occur in the presence of a variety of E2s. However, it has been suggested that an additional, but ill-defined region of Nedd4 could also be important in determining E2 specificity [203]. The structure of the HECT domains from E6- AP and the Nedd4-family protein WWP1 have been determined [205, 206]. They were found to be composed of two lobes; the amino lobe interacts with E2s and the carboxyl lobe contains the catalytic cysteine residue, separated by a flexible hinge [205, 206]. In addition to the catalytic cysteine residue that forms a thioester link with ubiquitin, it was recently shown that a conserved phenylalanine in the HECT domain is also required for functional E3 activity [207].

3.3. Nedd4/Nedd4-like-mediated ENaC Regulation

In 1996 Staub et al. [63] using a yeast two-hybrid screen, found that the carboxyl terminal of βENaC interacts with rat Nedd4, which like mouse Nedd4 has three WW domains. This interaction was prevented by introduction of PY motif point mutations, similar to those attributed to Liddle's syndrome [63]. Since then many investigators have shown by various *in vitro* and *in vivo* approaches that ENaC PY motifs and Nedd4/Nedd4-like WW domains interact [14]. In particular, *in vitro* interaction of ENaC PY motif was shown with WW domains of Nedd4-1 [63, 208-217], Nedd4-2 [114, 159, 217], WWP1 [218], and WWP2 [219]. These interactions with ENaC were confirmed in intact cells by co-immunoprecipitation with Nedd4-1 [63, 220] and Nedd4-2 [115, 220-222]. Subsequently, evidence has accumulated that the PY motifs of β or γENaC interact with the WW domains 3 or 4 of human Nedd4-1 or Nedd4-2 [211, 216, 217, 220, 223].

Several groups have demonstrated that ENaC is a protein with a rapid turnover of both the total and the cell surface pool ($T_{1/2}$ = approx. 1 hour) [224-228]. Rapid turnover is a hallmark of ubiquitylated proteins; in the case of ENaC, this rapid turnover depends on both proteasomal and lysosomal degradation [227-229]. It has been shown using ENaC with cytoplasmic lysines mutated to arginine, hence with mutated putative ubiquitylation sites, that ubiquitylation of ENaC controls its cell surface expression but not its intrinsic activity (*i.e.*, Po or single channel conductance [227]). So far there has been no direct demonstration that Nedd4/Nedd4-like proteins ubiquitylate ENaC.

It has been demonstrated by several approaches that Nedd4/Nedd4-like proteins, in particular Nedd4, Nedd4-2, or WWP2, can regulate ENaC. In mouse mandibular cells, it is possible to interfere with Na^+ feedback regulation by adding anti-Nedd4 antibodies or purified WW domains from Nedd4-1 or Nedd4-2 into the pipette of a whole-cell patch-clamp set-up and letting them diffuse into the cell [93, 208, 210, 223]. Such data have been interpreted as evidence that either Nedd4 or Nedd4-2 is involved in Na^+ feedback regulation in mandibular cells. Strong support for an involvement of Nedd4/Nedd4-like proteins in ENaC regulation comes from experiments done in the *Xenopus laevis* oocytes system, in which regulation of ENaC by co-expressed Nedd4-1 [94, 211, 215], WWP2 [219], and Nedd4-2 [220, 221, 230] has been demonstrated. The strongest effects are seen with Nedd4-2.

Overexpression of Nedd4-2 leads to the nearly complete suppression of amiloride-sensitive Na^+ channels, an effect that is dependant on the PY motifs of ENaC [220, 221, 230]. Conversely, catalytically inactive Nedd4-2 mutants increase these currents, most likely by competing against endogenous Nedd4-2. Moreover, Nedd4-2 has been shown to control cell surface expression of ENaC [230].

3.4. The Tissue distribution of Nedd4

Nedd4-2 expression is found in various tissues and cell types, suggesting that Nedd4-2 has multiple cellular functions [231]. Northern blot analysis revealed Nedd4-2 mRNA expression in brain, heart, lung, liver, and kidney [220, 221, 232]. Within the human kidney, Nedd4-2 mRNA was reported to be expressed along the entire nephron [233]. Loffing-Cueni et al. [231] confirm these data for the mouse nephron and extend them to the protein level by demonstrating localization of Nedd4-2 protein in several tubular portions. The highest expression levels of Nedd4-2 protein were seen in ASDN segments, consistent with the proposed regulatory role of Nedd4-2 for ENaC function. However, Nedd4-2 also was seen in tubular segments that do not express ENaC. In particular, PT cells showed a considerable amount of Nedd4-2 mRNA expression and protein immunostaining.

3.5. The Deubiquitylating Enzyme Family (DUB)

In addition to E1, E2s and E3s, ubiquitylation can be directly regulated by the effect of other proteins. A well characterised family of such proteins is that of the deubiquitylating enzymes (DUBs). On the basis of sequence comparison and enzymatic specificity, the DUB group can be divided into two families, the ubiquitin C-terminal hydrolases (**UCH** or UCH type-1) and the ubiquitin-specific processing proteases (**UBP** or UCH type-2).

UCH enzymes are generally smaller in size (approx. 200 amino acids), are closely related in sequence, homologous to the cathepsin B cysteine proteases, are classified within the C12 peptidase family [234] and efficiently cleave small C-terminal extensions and adducts from ubiquitin [235-237]. They specifically remove small peptides and proteins from the C-terminal Gly-76 of ubiquitin. Their function is to re-cycle ubiquitin monomers for ligation to new substrates. UCHs are

developmentally regulated and display tissue-specific expression [238, 239].

In contrast, UBPs range in size from 50 to 300 kDa and apart from the catalytic domains, UBPs can be extremely divergent, suggesting that they function in a substrate-specific manner [174, 240]. The UBP enzymes, classified within the C19 peptidase family, are also involved in ubiquitin re-cycling, but their activity is directed towards the disassembly of various forms of polymeric ubiquitin or ubiquitin-like protein complexes [241].

UCHs are known to be required for processing ubiquitin-precursor proteins, whereas UBPs are purported to catalyze deconjugation of ubiquitin from larger protein substrates, and some members efficiently disassemble polyubiquitin chains [238, 242]. There is evidence for certain DUBs functioning to stabilize proteins by deubiquitylation, while DUB function is also attributed to ubiquitin cleavage from proteins during proteasomal degradation, resulting in the recycling of ubiquitin [174].

Nearly 20 DUBs have been identified in yeast, and the human genome is purported to contain more than 90 [172, 243]. The sheer number of DUBs contained in the genome suggests a potential for selective deconjugation of ubiquitin. Indeed, specific deubiquitylation of p53, a tumor suppressor protein whose expression is regulated by ubiquitylation, has been reported [244-246]. Also, type 2 iodothyronine deiodinase is specifically deubiquitylated by von Hippel-Lindau protein-interacting deubiquitylating enzyme-1 [247]. Additionally, inactivation of individual DUBs produces distinct phenotypes; the Drosophila *faf* gene is required for normal eye development [248], mutations in CYLD predispose individuals for cylindromatosis [249], and cyk-3 mutants in *Caenorhabditis elegans* are defective in osmotic regulation and cytokinesis [250].

3.5.1. The USP family

Ubiquitin-specific proteases (USPs) are a large family of cysteine proteases, capable of cleaving both linear ubiquitin precursor proteins and posttranslationally formed ubiquitin–protein conjugates, and are the mammalian homologs of the yeast UBP family [251-253]. These enzymes contain two highly conserved sequence motifs that were discovered during sequence alignment of the first three yeast UBPs [251]. These are the “Cys box” and the “His box,” which contain a conserved cysteine and histidine residue, respectively, that were proposed to form part of the active site of these thiol proteases [251], consistent with the results of subsequent mutagenesis [248, 254-258] and structural [243] studies. The region between the Cys box and the His box defines the catalytic core responsible for deubiquitylating activity of the enzyme. USPs also contain N-terminal or/and C-terminal extensions of varying length and unique sequence. The functions of these sequences remain poorly understood, although the extensions and insertions have been suggested to function in substrate recognition, subcellular localization of the enzymes, and protein-protein interactions. In addition to processing of Ub precursors and salvage of trapped Ubs, UBPs are responsible for removing Ub from polyubiquitylated proteins and for disassembly of free poly-Ub chains. Large family members of UBPs have been identified in a variety of organisms, which appear to regulate a diverse set of biological processes.

Humans contain at least 28 USPs that have been characterized to varying extents [241, 252, 259], with a total of 50 suggested by analysis of the human genome sequence. The mouse genome would be expected to contain a similar number. It is proposed that the presence of such a large family of USPs in all eukaryotes is not required merely for the cleavage of ubiquitin precursors, and it is indicative of other regulatory roles in the ubiquitin pathway, where variable N-terminal extensions might be responsible for specific functions in selecting and binding substrates, and/or mediating localization of the enzyme [240, 241, 259].

4. Na^+ transport and Diseases

4.1. Hyperaldosteronism

In primary hyperaldosteronism aldosterone is produced in excess, e.g. due to an adenoma of aldosterone producing cells. In contrast, secondary hyperaldosteronism is the result of an increase in aldosterone secretion that is due to an external stimulus, e.g. renin overproduction. The symptoms of both conditions are similar: Hypernatremia (water loss exceeding sodium loss), hypokalemia (lowered potassium plasma levels) and alkalosis (lowered plasma proton concentration) leading to hypervolemia and hypertension.

4.2. Pseudohypoaldosteronism

Autosomal dominant and recessive forms of pseudohypoaldosteronism type 1 (PHA1) are recognized. Patients suffering from this disease are resistant to the action of aldosterone but have markedly elevated plasma levels of aldosterone and renin. Major symptoms of PHA1 are hypotension (salt wasting) and hyperkalemia. The autosomal dominant form of PHA1 is characterized by a loss of the aldosterone effect due to mutations in the mineralocorticoid receptor gene [260, 261]. In contrast, the autosomal recessive form of PHA1 is caused by a “loss of function mutation” in any of the three subunits of ENaC. These mutations were originally described as truncations, frame shifts or missense mutations causing one amino acid substitution in the intracellular N-terminal region (α- and β-subunit) and in the extracellular loop (α- and γ-subunit) of the subunits. The missense mutation G37S in the N-terminal region of the β-subunit has been described to cause a dysfunctional channel postulating two basic mechanisms, defective protein trafficking and/or defective gating of the channel [262]. A second instructive missense mutation the C133Y in the extracellular loop of the α-subunit has been reported to lead to a reduction of Na^+ transport caused by a less efficient transport of assembled Na^+ channels to the plasma membrane [263].

4.3. Liddle's Syndrome

Liddle's syndrome is a rare autosomal dominant disorder elicited by mutations of β or γENaC subunit that result in a gain of function. The involved mutations were originally described as truncations or frame shifts in the C-termini of β or γENaC subunits [60, 264]. More detailed studies revealed, that these mutations delete a critical proline-rich region (PPxY-motif) of the C-terminal region of the protein that interacts with the protein ubiquitin ligase called Nedd4 [230]. A proposed mechanism is that the mutations prevent Nedd4 binding and thereby lead to a defective internalization of mutant ENaC [63]. This increases ENaC activity, as observed at the level of *Xenopus laevis* oocytes and Madin-Darby canine kidney cells [265, 266]. The symptoms of Liddle's disease resemble primary hyperaldosteronism and severely suppressed plasma renin and plasma aldosterone levels.

4.4. Gitelman's and Bartter's Syndrome

Both diseases are caused by autosomal recessive mutations and produce distinctive clinical and physiological pictures of hypokalemic alkalosis. Molecular genetic approaches have demonstrated that mutations in the gene encoding the thiazide-sensitive Na^+-Cl^- cotransporter cause the Gitelman's syndrome. Bartter's syndrome appears to be a collection of several monogenic diseases. The cause is the lack of Na^+ reabsorption in the TAL due to the dysfunction of either the apical bumetamide-sensitive Na^+-K^+-$2Cl^-$ cotransporter [267], the basolateral chloride channel [268, 269] or the apical potassium channel [270], which are all located in the TAL.

The first three diseases described in this chapter concern the ASDN and thus indicate the importance of aldosterone regulated Na^+ reabsorption in this part of the kidney tubule for the control of blood pressure.

Materials and Methods

Animal studies were in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Science, Bethesda, MD).

1. Mouse Treatment, Kidney Dissection and Tubule Microcollection

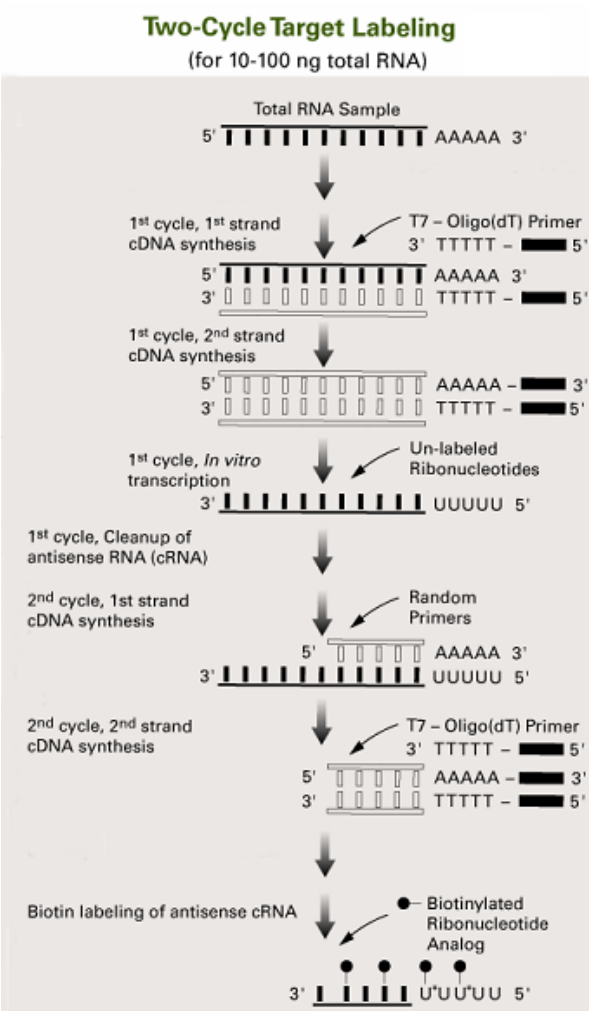
Female C57BL/6JOlaHsd mice (Harlan), approximately 8 weeks old, received drinking water containing 0.3% NaCl for at least 5 days prior to the treatment. All mice were housed in a temperature- and humidity controlled room with an automatic 12 h light/dark cycle (6 AM/ 6 PM). Aldosterone (Acrôs Organics) (10 μg/kg body weight in 0.33% ethanol, 0.9% NaCl) or canrenoate (Sigma) (10 mg/kg in 0.9% NaCl) was injected intraperitoneally or mice left uninjected. Forty two minutes later, mice were anesthetized with ketamine (Vétoquinol AG) and xylasine (Streuli AG) (intraperitoneally 100 μg/g and 0.5 μg/g, respectively). They were then perfused through the left ventricle with Ringer solution containing collagenase and protease (5 mg and 1 mg in 15 ml, respectively) (Sigma) while the lower vena cava was opened. Kidneys were removed and cut transversally in 2 mm slices that were incubated for 10 min at 38°C in a collagenase solution (1 mg/ml Ringer solution). These tubule preparations were then transferred to ice-cold Ringer (1 h after aldosterone injection), washed and resuspended three times. Segments (75-85) of CCD/CNT (approx. 1,1 mm of length) were identified under a binocular microscope (SMZ1000, Nikon) at 4°C and transferred directly into RLT lysis buffer of the Qiagen RNeasy Micro Kit (Qiagen) containing 1% β-mercaptoethanol (Sigma-Aldrich) using a pair of sharp forceps (F.S.T. GmbH). For plasma aldosterone and corticosterone measurements, mice were anesthetized as above and blood collected by puncture of the vena cava. Plasma aldosterone and corticosterone levels were measured using an ELISA- and a ^{125}I-RIA-kit from IBL (Immuno-Biological Laboratories), respectively.

2. RNA Purification and Quality Control

Total RNA from microcollected CCD/CNT was purified using RNeasy Micro Kit (Qiagen). The RNA samples were tested for quality using an Agilent 2100 Bioanalyzer® (PicoChip®, Agilent Technologies) and for quantity using the ND-1000 NanoDrop® UV-spectrophotometer (NanoDrop Technologies). Only samples showing a 28S/18S ribosomal RNA ratio ≥ 1 or a RNA Integrity Number (RIN; Agilent Technologies) >7 (range 1-10 with 10 for best RNA quality) were used for target cRNA preparation.

3. Microarray Target cRNA Preparation

RNA amplification and labeling was performed according to a two-cycle cDNA synthesis protocol.:



Briefly, ≥ 30 ng of total RNA was concentrated to a volume of approximately 3 µl using a speed-vac system and used for first and second strand cDNA synthesis with the GeneChip® Two-Cycle cDNA Synthesis Kit (Affymetrix) in the presence of an oligo-dT primer containing a T7 RNA polymerase promoter site. cRNA was prepared by in vitro transcription using the MEGAscript T7 Kit (Ambion). The resulting cRNA was used for a second round of first-strand cDNA synthesis with random hexamer primers, followed by second strand cDNA synthesis with the same kit as above. Labeled target cRNA was then prepared in a second round of in vitro transcription using

biotin-labeled UTP (GeneChip® IVT Labeling Kit, Affymetrix). After each round of amplification the cRNA were tested for quality using an Agilent 2100 Bioanalyzer® (PicoChip®, Agilent Technologies) and for quantity using the ND-1000 NanoDrop® UV-spectrophotometer (NanoDrop Technologies). Only samples showing labeled cRNA average size distribution higher than 0.5 kb were accepted for hybridization.

4. Target cRNA Hybridization and GeneChip® Scanning

Biotin-labeled cRNA samples (15 μg) were fragmented randomly to 35-200bp at 94°C for 35 min in presence of Fragmentation Buffer (Affymetrix) and were mixed in 300 μl of hybridization buffer containing Eukaryotic hybridization control mix (Affymetrix) and Control Oligo B2 (Affymetrix), 0.1 mg/ml herring sperm DNA and 0.5 mg/ml acetylated bovine serum albumin in 2-(4-morpholino)-ethane sulfonic acid (MES) buffer, pH 6.7, before hybridization to GeneChip® MOE 430 2.0 arrays. Hybridizations were carried out at 45°C for 16 h at constant rotation (60 rpm). Subsequent washing and staining steps were performed using the Affymetrix Fluidics Station 450 EukGE-WS2v5_450 washing protocol (Affymetrix). Stained arrays were scanned using an Affymetrix® GeneChip® Scanner 3000 (Affymetrix), and the probe set intensity values for each gene were calculated by Affymetrix GeneChip® Operating System Software (GCOS 1.0, Affymetrix).

5. Real-Time RT-PCR

Samples of 3 μl RNA (out of 60 μl) were used as templates for reverse transcription using TaqMan® Reverse Transcription Kit (Applied Biosystems) in the presence of 2.5 μM random hexamer primers (Applied Biosystems). Real-time PCR was performed using 3.5 μl of the cDNA as template using the TaqMan® Universal PCR master mix (Applied Biosystems). Primers and probes were designed using Primer Express 2.0 software (Applied Biosystems) to result in amplicons of 70-100 bp that span intron-exon boundaries. Each primer pair (Table 1.) was first tested on mouse kidney cDNA and resulted in a single product of the expected size (data not shown). Probes (Table 1.) were labelled with reporter dye

FAM at the 5' end and the quencher dye TAMRA at the 3' end (Microsynth). Reactions were run in 96-well optical reaction plates using a Prism 7700 cycler (Applied Biosystems). Thermal cycles were set at 95°C (10 minutes) and the 40 cycles at 95°C (15 seconds) and 60°C (1 minute) with auto ramp time.

Table 1. Primers and probes used for real-time RT-PCR

gene	sense primer (5'-3)'	probe (5'-3')	anti-sense primer (5'-3')
Grem2		Mm00501909_m1*	
Sgk1	GAG GGA GCG CTG CTT CCT	ACC ACG GGC TCG ATT CTA CGC AGC	GCA GAT AGC CCA GGG CAC T
Atf3	AGA CCC CTG GAG ATG TCA GTC A	CAA GTC TGA GGC GGC CCC TGA A	CGC CTC CTT TTC CTC TCA TCT T
Crem	TTG GGA ATA CCA GGC ACA TCA	AAA GTT CAT TCA GTA TTT TTC TAT ATG AGG GTC TTC GTG A	TGA TTC GCA TAA ACG TAG AGA AAT TCT
Usp2	TCT GTC CCC GTC CCT GC	TGC TCA ACA AAG CCA AGA ATT CAA AGA GTG C	AAG GTT TCG AAG ACC AGC CAG
H^+/K^+-ATPase		Mm00446786_m1*	
Slc22a8	TGC TTG GAT GGC TGG ATC TAC	CAT TGT GAC AGA GTG GGA CTT GGT ATG CG	TGC CAT CTC CTT CAG TTT GTT G
G0s2	AAG TGC TGC CTC TCT TCC CAC	CACA CCT AGG CCC AGC CAC CCA	TTC CAT CTG AGC TCT GGG CT
β-actin	CCA CCG ATC CAC ACA GAG TAC TT	AGG AGG AGC AAT GAT CTT GAT CTT CAT GG	GAC AGG ATG CAG AAG GAG ATT ACT G
αENaC	GGT GCA CGG TCA GGA TGA G	CTG CTT TTA TGG ATG ATG GTG GCT TCA A	TAG TTG CCT CCG AGG CTG TC
βENaC	TCC TAG CTT GCC TGT TTG GAA	AGA GCC CTG CAG TCA TCG GAA CTT CA	CAG TTG CCA TAA TCA GGG TAG AAG AT
NKCC2	TCA CCA CCG TGG CCT ACA TA	CTA TTT GCG TAG CCG CCT GTG TGG TC	GGT GCA CGG TCA GGA TGA G

* Applied Biosystems TaqMan® Gene Expression Assays ID

To analyze the data, the threshold was set to 0.06 (value in the linear range of amplification curves). All reactions were run in triplicates and the abundance of the target mRNAs was calculated relative to a reference mRNA (mouse β-actin). Assuming an efficiency value of 2 (fold-increase in input mRNA required to decrease the cycle number by 1), relative expression ratios were calculated as $R = 2^{(Ct(\beta\text{-actin}) - Ct(test))}$, where Ct is the cycle number at the threshold and test stands for the tested mRNAs. Based on this analysis, some samples (<1/3) were excluded from microarray target RNA synthesis because of high $Na^+/K^+/2Cl^-$-cotransporter (Nkcc2) contamination, a relatively low enrichment in α- and βENaC mRNA or, for aldosterone-treated mice, a Sgk1 upregulation <2-fold. Real-time RT-PCR was performed similarly for controlling the regulation of 8 mRNAs considered as highly regulated by microarray.

6. Quality Control of Microarrays and Statistical Analysis

Per condition, six GeneChips® were used for hybridization. Each hybridization was performed with target RNA from individual mice. Array images were inspected visually for artefacts and B2 Oligo performance. Raw data processing was performed using the Affymetrix Gene Chip Operating System Software (GCOS 1.0, Affymetrix) and the GCRMA preprocessor of the Genespring software 7.1 (Silicongenetics), respectively. Summarization for the respective probe sets giving rise to the actual expression values was carried out in two independent approaches using different algorithms; MAS5 and GCRMA.

For the first approach the MAS5 algorithms was employed as detailed in Hubbell, E. et al. [271]). In order to compare the expression values of the genes from chip to chip, global scaling was performed, which resulted in the normalization of the trimmed mean of each chip to a target intensity (TGT value) of 500. The average background and noise (Raw Q) ranges from 40 to 100, and 1 to 5, respectively. Absent and present calls (>50%) were based on detection *P*-values calculated by application of a signed-rank call algorithm [272]. Optimal 3'/5' hybridization ratios (below 7) for the housekeeping genes (GAPDH and β-actin) and (around 1) for the spike-in controls (BIOB, BIOC, CREX, BIODN) added into the hybridization cocktail, were taken into consideration. The second algorithm applied for the probe

set summarization was the GeneChip Robust Multichip Average (GCRMA, [273]) as implemented in Genespring 7.1. GCRMA corrects for background noise, as well as non-specific binding. Quantile normalization was used to adjust the PM probe intensity distributions among all GeneChip measurements and summarization of the probe intensities into expression measures was carried out using a median polishing estimation method.

To assess the significance of differential gene expression between the conditions tested, we applied statistical parametric methods based on the comparisons between the groups of replicates analyzed. Unreliable genes were filtered out using the absent/present calls resulting from the MAS5 analysis. Unequal variance t-test was applied to detect genes that were significantly differentially expressed with a significance level of $P \leq 0.05$. Finally, the resulting significant genes were filtered for fold changes between the conditions of ≥ 2.

7. Prediction of Mineralocorticoid Hormone Response Elements in the Promoter Region of the Mouse Usp2-45 Gene

Consensus steroid hormone response elements contain symmetric imperfect direct, palindromic or inverted palindromic (everted) repeats of hexameric halfsite sequences such as 5'-AGAACA-3' that are usually divided by 3 bp-long spacers. In order to predict potential binding sites for mineralocorticoid hormone response elements, we analyzed the promoter region -6.5 kb upstream of the ATG start codon of the mouse Usp2-45 gene by using the computational statistic models Position Weight Matrix (PWM) and Hidden-Markov-Model (HMM) implemented in the web-based program Tiger SHRE Finder 1.0 (http://birc.ntu.edu.sg/%7epmaria/ [274]. Within this region we found 13 HRE binding sites predicted by PWM (similarity coefficient bigger than 0.85) and 5 HRE by HMM (similarity coefficient bigger than 0.33). For better sensitivity, accuracy and avoiding false positive results both methods were taken together and 4 HRE sequences were predicted by both methods (Figure 17.).

8. Western Blotting of Usp2-45 in Microcollected Mouse Kidney Connecting Tubule and Collecting Duct

Mouse treatment, kidney dissection and tubule microcollection were performed as described above for RNA preparation, but tubule segments (75-85) were transferred into RIPA buffer for lysis. Two µg total proteins were loaded per lane on a 10% SDS-polyacrylamide gel and subsequently transferred electrophoretically to a PVDF-membrane (Immobilon-P, Millipore). Blots were blocked and incubated with primary antibody as described below under "Western blot in mpkCCD$_{c14}$ Cells" using 10 µg/ml anti-Usp2-45 antibody and monoclonal anti-β-actin antibody (1:5000, Sigma). In the peptide competition experiment the primary antibody was preincubated for 1 h at room temperature with the immunizing peptide at a 1:100 molar ratio (AB:peptide). The anti-Usp2-45 antibody, blot incubation conditions and washes are described below in "Westen blot in mpkCCD$_{c14}$ Cells". The specific bands were quantified by using the AIDA Image Analyzer 3.44 program (raytest Inc.).

9. Cloning of Mouse Usp2-45, Usp15, Site-Directed Mutagenesis and cRNA Preparation

Mouse kidney or heart (Usp2-69) RNA was isolated with RNeasy Mini Kit according to the manufacturer's protocol (Qiagen) and reverse transcribed (oligoDT primers) using the TaqMan Reverse Transcription Kit (Applied Biosystem). The Usp2-45 (RefSeq No NM_198091) and Usp15 (RefSeq No NM_027604) cDNAs were amplified by PCR using 2.5 U of a proofreading polymerase (Pfu, Promega), 1x Pfu-reaction buffer (Promega) 2mM dNTPs, 0.5µM 5'-Primer, 0.5 µM 3'-Primer for 40 cycles (45 seconds 96°C, 1 minute 50-70°C, 5 minutes 72°C).

The primers for PCR included restriction sites for cloning (each 5'XhoI, HindIII3') and were ordered from Microsynth

(Usp2-45: 5′-AAAACTCGAGCCGCGTTGGGTTTGGGGCATAG-3′,
5′-AAAAAAGCTTGGGGCCACCACAGGGAAGAG-3′,
Usp15: 5′-AAAACTCGAGGCTAGTGGAAGAAGATGGCGGAAG-3′,
5′-AAAAAAGCTTGGGATTTATTTGGGATTTCCT-3′,
Usp2-69: 5′-AAAACTCGAGCCCATGAGGCTCCCAGTACC-3′,
5′-AAAAAAGCTTGGCCACCACAGGGAAGAGGGAAG-3′).

The amplified PCR fragments were sub/cloned in the pSDeasy vector. *Xenopus laevis* ENaC subunit ($\alpha\beta\gamma$*X*ENaC) and *Xenopus laevis* Sgk cDNAs (*X*Sgk) were used [266, 275]. For changing cysteine 67 of Usp2-45 to alanine the QuickChange® site directed mutagenesis kit (Stratagene) was used with following primer pair:

5′-AACCTTGGGAACACGGCTTTCATGAACTCAATT-3′;
5′-AATTGAGTTCATGAAAGCCGTGTTCCCAAGGTT-3′.

To generate the rENaC-Km mutant missing all potential ubiquitylation sites, all cytoplasmic lysines of rat α-, β-, or γENaC [58] were mutated to arginine using the Quickchange® kit (Stratagene). For cRNA synthesis, mUsp2-45, *X*Sgk, β*X*ENaC, αrENaC and βrENaC plasmids were linearized with BglII, α- and γ*X*ENaC with AflIII (Promega), γrENaC with PvuII and used as template for RNA synthesis with mMESSAGE mMACHINE™ kit (Ambion).

10. Coexpression of mUsp2-45, mUsp15, $\alpha\beta\gamma$*X*ENaC and *X*Sgk in Xenopus laevis Oocytes

Stage V and VI oocytes were injected with 10 to 36 ng of Usp2-45, Usp2-45mut, Usp2-69, Usp15, 0.005 ng of each α-, β- and γ*X*ENaC subunit and 5 ng of *X*Sgk cRNA in 100 nl of nuclease free water. Oocytes injected with $\alpha\beta\gamma$*X*ENaC cRNA were incubated for 2 days at 16°C in ND10 buffer (10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 86 mM *N*-methyl-*D*-glucamine (NMDG)-Cl and 5 mM HEPES, pH 7.4 adjusted with 1 M Tris, and 50 mg/l tetracycline), whereas oocytes not injected with *X*ENaC cRNA were kept in ND96 buffer (NaCl instead of NMDG).

11. Two-Electrode Voltage Clamp on Xenopus Oocytes

The two-electrode voltage clamp technique was used for the recording of whole-cell currents from *Xenopus laevis* oocytes at room temperature 40-48 h after injection with αβγ*X*ENaC, *X*Sgk, mUsp15, mUsp2-69 and mUsp2-45 cRNA using a commercial two electrode voltage-clamp (Oocytes Clamp OC725C, Warner Instrument Corp.) connected to data acquisition hardware (Digidata 11322A, Axon Instruments). A computer running pClamp8 software (Axon instruments) was used to control the clamp, the valves switching the solutions, and to record currents, as previously described [276]. For current-voltage (I-V) recordings, oocytes were clamped (Vh = -50 mV) and step changes in membrane holding potential were applied (-140 to +20 mV in 20 mV increments). At each membrane potential four pulses of 80 ms duration were averaged. Recordings were made in ND10 and ND96 solutions and 10 μM amiloride was added to the ND96 solution to obtain the amiloride-sensitive current. Calculations and graphs were made using GraphPad Prism™ Version 4.0 (GraphPad Inc.) and significance was tested using the Kruskal-Wallis or Mann-Whitney test if not stated otherwise.

12. Ubiquitylation of ENaC in Hek293 Cells

Hek293 cells, grown on 75 cm^2 dishes were transfected by the calcium phosphate technique with 4.5 μg of the plasmids encoding rat ENaC subunits (HA-tagged αENaC, βENaC [64], Flag-tagged γENaC [266]), 13.5 μg of Usp2-45 and 2 μg of hNedd4-2 (wild-type or inactive mutant; as indicated [277]). After 24 hours, cells were washed with 10 ml of PBS and dissociated in 1 ml of dissociation buffer (5% glycerol, 1 mM EDTA, and 1 mM EGTA, pH 8.0). Cells were recovered with 10 ml PBS, centrifuged for 2 min at 4°C and 1500 g and pellets frozen at -70 °C. Cells were lysed in 1 ml buffer containing 50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM EGTA, 10 % glycerol, 1 % Triton X-100, 1 mM dithiothreitol, 100 mM NaF, 10 mM di-Na-pyrophosphate, 1 mM PMSF, 1 x protease cocktail (Roche) and then centrifuged for 15 minutes at 21'500g (4°C). Supernatants were recovered and immunoprecipitations carried out with anti-HA antibodies (recognizing HA-tagged

αrENaC; Santa Cruz) or anti Flag antibodies (recognizing flag-tagged γrENaC; M2, Sigma) for 2.5 h. Immunocomplexes were recovered with protein G sepharose during 1.25 h at 4°C on a rotating wheel. Protein G sepharose was washed with washing buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM EGTA, 10 % glycerol, 0.2 % Triton X-100, 1.5 mM $MgCl_2$, and 1 mM PMSF) and the immune complexes recovered in SDS-PAGE sample buffer. The samples were analyzed on 8 % or 5-15 % SDS-PAGE and Western blotting using anti αrENaC, γrENaC antibodies [227], or anti-ubiquitin antibodies (FK2, Affinity).

13. Culture and Retroviral Transduction of mpkCCD$_{c14}$ Cells

The mouse kidney cell line mpkCCD$_{cl4}$ and its culture was originally described by Bens et al. [278]. For electrophysiological and RNA expression measurements, mpkCCD$_{cl4}$ cells were seeded respectively on 12 mm diameter Snapwell filters or 24 mm diameter 24-well plastic plates (Corning Costar). After 5–7 days of culture in standard medium and for electrophysiology an additional 2-7 days in filter medium (growth medium without serum, Apo-transferrine and EGF) cells were transferred into serum- and hormone-deprived medium 12 – 20 h prior to the experiments [279]. Because of the lack of sufficient mineralocorticoid receptor expression in mpkCCD$_{c14}$ cells, aldosterone was used at 0.3 or 1.0 x 10^{-6} M to obtain near-maximal effects via the glucocorticoid receptor [279].

Stable populations of mpkCCD$_{c14}$ cells expressing Usp2-45, and Usp-2-45mut were obtained according to the protocol provided on the website http://www.stanford.edu/group/nolan/ with slight modifications [280]. Briefly, ecotropic pseudoretroviral producer cells (Phoenix) were plated 24 h prior to transfection at 50% density on 60 mm plates. The next day, 5 μg of the retroviral construct encoding Usp2-45 or Usp2-mut were applied using FuGENE6 (Roche) according to the manufacturers recommendation. The medium was replaced after 24 h and the cells subsequently kept at 32°C. The pseudoretrovirus-containing supernatant was harvested after 24 h and filtered through a 45 μm sterile filter. After addition of Polybrene at a final concentration of 4 μM, the entire supernatant (3 ml) was given to 5% confluent mpkCCD$_{cl4}$ cells plated the day before on 6-well plates (35 mm diameter). The cells were centrifuged at 130 g and 32°C for 30 min

and then kept for 6 h at 32°C (5% CO_2) before the supernatant was replaced by usual medium. The procedure was repeated the next day using fresh retroviral supernatant harvested from the same Phoenix cells. A transduction efficiency of 70–100% was achieved when using EGFP (enhanced green fluorescent protein) as a marker. This protocol was repeated 2 times to achieve maximal expression. The resulting multiclonal cell populations were then kept under selective pressure with 1 μg ml^{-1} puromycin.

14. Western Blotting and Real-Time PCR of Usp2-45 in mpkCCD$_{c14}$ Cells

An antibody selective for the Usp2-45 isoform of mouse Usp2 was raised against a NH_2-terminal epitope (MRTSYTVTLPEEPAAC) (Pineda) and affinity purified on antigen peptide using the SulfoLink® Kit (Pierce/Perbio) according to the manufacturer protocol. The antibody against both, Usp2-45 and Usp2-69 was ordered from Abgent (Cat.Nr.:AP2131c) mpkCCD$_{c14}$ cells were harvested from plastic cultures and lysed in RIPA buffer (150mM NaCl, 1% IPGAL, 0.5%DOC, 0.1% SDS, 50mM Tris, pH7.5) by passing them 10 times through a 25 G needle and *Xenopus* oocytes were lysed in buffer (10 μl per oocyte) containing 250 mM sucrose, 0.5 mM EDTA, 5 mM Tris-HCl, pH 6.9, 1 mM PMSF, and 1 mg/ml of leupept also by passing them 10 times through a 25 G needle. Oocyte debris were pelleted by two consecutive centrifugations at 100 g for 10 min. Equal amounts of proteins derived from mpkCCD$_{c14}$ cells and a smaller amount from oocyte expressing Usp2-45, Usp2-69 and/or ENaC were loaded on a 10% SDS PAGE and subsequently transferred electrophoretically to a PVDF-membrane (Immobilon-P, Millipore). After blocking with 3-5% milk in Tris-buffered saline/ 0.1% Triton X-100 (Sigma-Aldrich) overnight at 4°C or 2 h at room temperature, the blots were incubated with primary antibody (10 μg/ml anti-Usp2-45) for 2 h at room temperature or overnight at 4°C. After washing and subsequent blocking for 1 h at room temperature, blots were incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:10,000; BD Transduction Laboratories) for 1 h at room temperature. Antibody binding was detected with the Super-Signal1 West Pico Substrate (Pierce). Chemiluminescence was detected

with a DIANA III camera (Raytest). RNA extraction and TaqMan® real time RT PCR were performed as indicated for mouse kidney and tubules. The Usp2 isoform-specific primers and probes are indicated in Table 1.

15. Transepithelial Electrophysiological Measurements

A transepithelial resistance of 2 kOhm x cm^2 was considered as prerequisite for using the filter cultures for further electrophysiological measurements. Filters were mounted in a multichannel voltage current clamp device (Model VCC MC6 Revision B, Physiological Instruments) and monolayers maintained under current clamp conditions at a DC clamp level of 0 μA. Bipolar pulses of 5 μA were given for 620 ms every 60 sec. After 90 min, aldosterone (300 nM) was added to the medium. The ENaC-mediated sodium current was specifically blocked by 10 μM amiloride after another 150 min and calculated as the difference in short circuit current before and 15 min after amiloride addition.

Results

Beforehand presenting the results of this study I would like to note the contribution to this work by Gabriele Adam for the *Xenopus* oocytes and Olivier Staub and collegues for the Hek293 cells expression studies.

The results presented in this following section have been published in the Journal of American Society of Nephrology (JASN) in March 2007.

Early Aldosterone-Induced Gene Product Regulates the Epithelial Sodium Channel by Deubiquitylation

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(J Am Soc Nephrol 18: 1084–1092, 2007)

** both authors contributed equally to this work*

1. Microarray to Identify Early Aldosterone-Regulated mRNAs in Mouse ASDN

To identify other early aldosterone-regulated gene products in kidney target cells in vivo, we first established a short-term aldosterone treatment scheme in mice. Female C57BL/6J mice receiving 0.3% NaCl in their water to partially repress their endogenous aldosterone production were injected at 10:00 AM (4 hours after begin of daytime) with aldosterone (10 μg/kg body weight, Figure 14.A and B), or not injected.

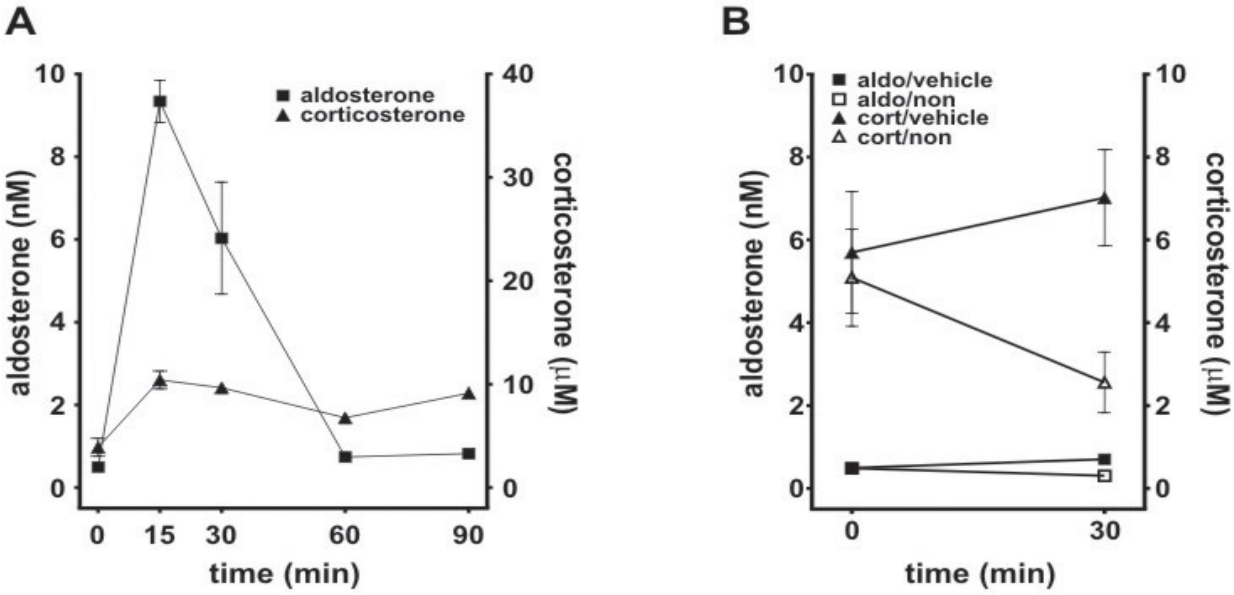

Figure 14. Conditions of short-term aldosterone stimulation. (**A**) Time-course of plasma aldosterone and corticosterone levels after intraperitoneal aldosterone injection (10 μg/ kg body weight) at 10 AM (mean ± SEM, n = 3 mice per time point). (**B**) Comparison of plasma aldosterone (aldo) and corticosterone (cort) at 10 AM and at 10.30 with or without (non) injection of vehicle at 10 AM (mean ± SEM, n = 3 - 6 mice per point).

The aldosterone injection led to a transient increase in plasma aldosterone which 15 min after injection was at a level of ~10 nM, a value within the range expected in low salt diet [281]. The plasma aldosterone concentration was nearly back to the initial value of approximately 0.5 nM after 60 min.

To obtain kidney aldosterone-target tissue RNA 1 h after hormone injection, mice were anesthetized 42 min after aldosterone injection and rapidly perfused with collagenase solution. Kidneys were removed, sliced and further digested in collagenase solution at 38°C for the rest of the hour (in the absence of aldosterone). The tubule preparation was then transferred to 4°C and segments of connecting tubule and cortical collecting duct (CNT/CCD) microselected.

The RNA samples prepared from single mice were tested in terms of quantity, integrity, enrichment, contamination and response to aldosterone (Figure 15.C and D).

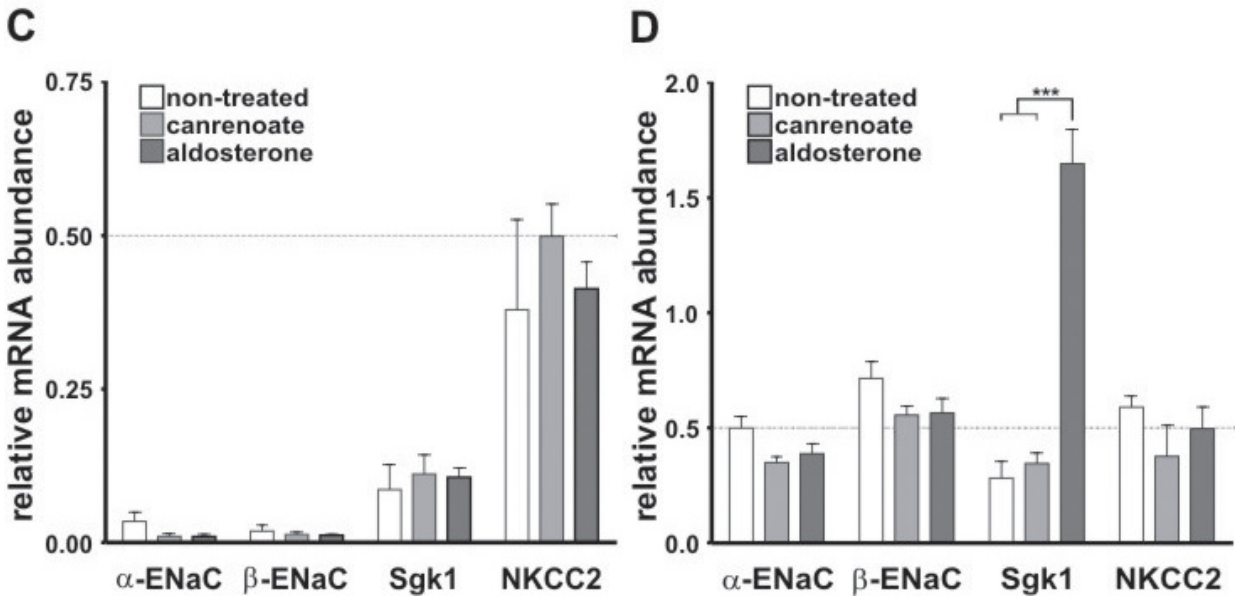

Figure 15. (**C**) and (**D**) Test for enrichment and aldosterone stimulation of RNA prepared from microselected CNT/CCD. Mice were injected with aldosterone as in (A), canrenoate or not injected and RNA prepared from tubules obtained after 1 h. The abundance of α-, βENaC, Sgk1 and Nkcc2 mRNAs was measured by real-time RT-PCR in RNA prepared from total kidney (**C**) and from microselected tubule segments (**D**). Given are means relative to β-actin ± SEM, n = 6 mice. ***P<0.0001 by 1 way-ANOVA.

These measurements revealed that in microisolated CNT/CCD segments ENaC mRNA was enriched 15 - 40-fold over total kidney, whereas thick-ascending limb-specific triple cotransporter Nkcc2 mRNA was not increased. Sgk1 mRNA was enriched 3 - 4-fold over the total kidney preparation and specifically further increased 5 - 6-fold by aldosterone. Interestingly, the mere manipulation of the mice increased plasma corticosterone slightly (Figure 14.A and B) but did not impact on Sgk1 expression (Figure 15.C and D). More than two third of the prepared RNA samples fulfilled the selection criteria of quantity, integrity, enrichment, lack of contamination and SGK1 induction and could be used as template for cDNA amplification and labelled target RNA synthesis. Target cRNAs made from 6 control and from 6 aldosterone-injected mice were separately hybridized to mouse full genome GeneChip® microarrays (MOE 430 v2, Affymetrix). Raw array data were processed using the GCRMA and the MAS5 algorithms (Microarray data are available at Array Express with the accession number E-TABM-229.). The data were filtered using a *t*-test with a cut-off *P*-value of $P \leq 0.05$ and using a fold-change limit of 2.0. The cumulative list of genes that fulfilled these criteria after processing with one of the algorithms comprised 90 genes (73 genes with GCRMA and 39 with MAS5). A scatter plot and the hierarchical clustering of the results calculated by the GCRMA method are shown in Figure 16..

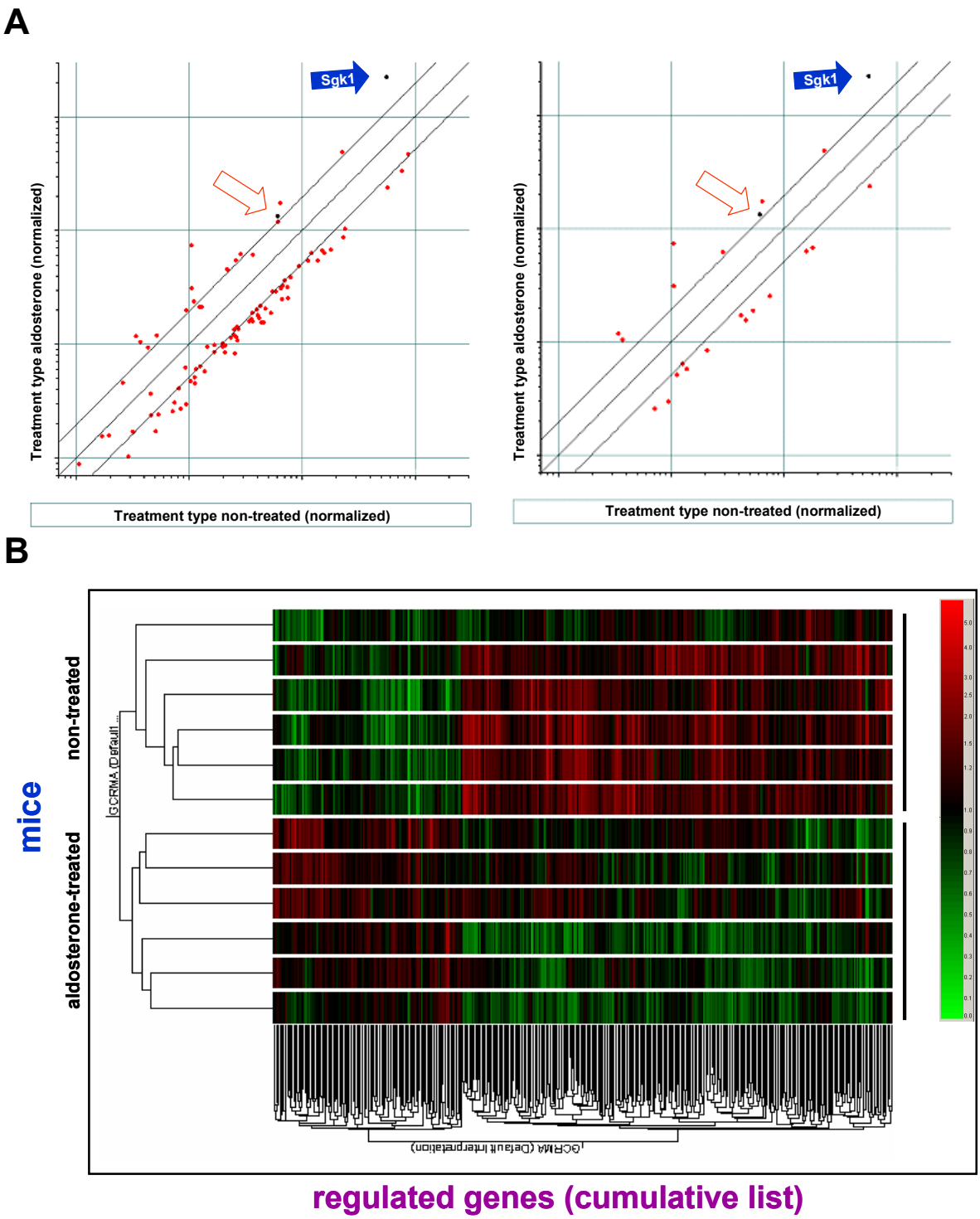

Figure 16. Scatter plot and hierarchical clustering of aldosterone-regulated mRNA. (**A**) Scatter plot calculated by the GCRMA method. The left panel shows the expression values (GCRMA values) of the 90 gene products of the cumulative list (2-fold regulation, $P<0.05$, with GCRMA or MAS5 algorithm). The right panel shows the 22 genes that fulfill the selection criteria for both methods. The expression values range within more than two orders of magnitude and the absolute highest signal is that of the Sgk1 mRNA. (**B**) Hierarchical clustering of the tested mice based on the cumulative list of genes. This graphical view of the GCRMA data visualizes the individual variability of expression of some mRNAs in particular in the non-treated mice.

As detailed in Table 2, 22 genes fulfilled these criteria with both algorithms, 9 that are upregulated and 13 downregulated. We retested the differential expression level of 8 mRNAs identified in the microarray screen by real-time PCR (see primers and probes sequences in Materials and Methods) on RNA from three other mice per condition. As shown in Table 2, the regulation was verified in 6 cases.

Table 2. Kidney CNT/CCD RNAs changed ≥ 2-fold by 1 h aldosterone

GeneChip		Affymetrix®			real-time PCR	
RNA*	**Genebank Acc. No.**	**fold change ‡**	**range**	***P*-value**	**fold change §**	***P*-value**
Upregulated genes						
protein related to DAN and cerberus†	NM_011825	7.2	2.1 - 17.7	0.045	5.6	0.029
serum/glucocorticoid regulated kinase	NM_011361	4.0	3.0 - 5.1	3.4E-05	8.1	0.001
activating transcription factor 3	BC019946	3.5	2.5 - 4.9	0.001	8.6	0.018
cAMP responsive element modulator	NM_013498	3.0	1.3 - 4.5	0.004	1.1	0.651
Mus musculus transcribed sequences	AI098139	2.9	1.7 - 3.6	1.4E-04		
RIKEN cDNA clone:6430570G24	BG069663	2.8	1.7 - 4.4	0.004		
ubiquitin specific protease 2	AI553394	2.2	1.2 - 3.8	0.019	1.6	0.001
growth arrest and DNA-damage-inducible 45 gamma	AK007410	2.2	1.5 - 3.2	0.001		
RIKEN cDNA clone:6430570G24	BG069663	2.2	1.5 - 2.9	8.7E-05		
Downregulated genes						
RIKEN cDNA 4833417L20 gene	AK017914	0.5	0.3 - 0.9	0.014		
Similarity to pir:A45973 (H.sapiens)	AI597080	0.5	0.3 - 0.6	0.009		
argininosuccinate synthetase 1	BM213298	0.4	0.3 - 0.5	0.007		
RIKEN cDNA clone:D630044A17	BB498095	0.4	0.2 - 0.6	0.006		
partial mRNA for hypothetical prot.	BG817292	0.4	0.3 - 0.6	0.006		
fatty acid binding protein 1, liver	NM_017399	0.4	0.2 - 0.8	0.006		
transcribed sequences	BE991175	0.4	0.2 - 0.6	0.006		
RIKEN cDNA clone:C230084M03	BM123508	0.4	0.2 - 0.8	0.005		
RIKEN cDNA 4833417L20 gene	BM217861	0.4	0.3 - 0.4	0.004		
H^+/K^+-ATPase alpha, nongastric	NM_138652	0.4	0.1 - 0.8	0.004	1.0	0.946
Slc22a8 (Oat3)	AV330315	0.3	0.1 - 0.6	0.004	0.1	0.024
G0/G1 switch gene 2	NM_008059	0.3	0.2 - 0.6	0.003	0.2	0.015
RIKEN cDNA clone E030031K24	BB533736	0.3	0.2 - 0.5	0.003		

*RNAs with fold-change ≥ 2 and *t*-test *P*-value $P \leq 0.05$ using GCRMA and MAS5 algorithms
†RNAs indicated in italic were also tested by real-time RT-PCR
‡Values obtained using GCRMA
§Real-time PCR data obtained with specific intron spanning primer/probe sets, n = 3 mice

2. Aldosterone-Induced Usp2-45 Deubiquitylates ENaC

Ubiquitylation of ENaC had previously been shown to play an important role for the control of Na^+ reabsorption [115] and the present screening had confirmed that Sgk1, a kinase known to inhibit the action of the ubiquitin-protein ligase Nedd4-2 on ENaC, was rapidly induced by aldosterone. We focussed now on Usp2, an ubiquitin-specific protease that was also induced and belongs to a large protein family involved in reversing the ubiquitylation of target proteins [240, 282, 283]. We specifically investigated the potential role of the isoform Usp2-45 that we detected in kidney by RT-PCR, unlike the other isoform Usp2-69 that is expressed only at low levels in the kidney [284] (Fakitsas and Verrey, unpublished observation) and in mpkCCD$_{c14}$ cell (10-fold difference, see below). The analysis of the promoter region around the TATA box of the Usp2-45 isoform identified at least 4 potential hormone response elements that support the possibility of a direct regulation by aldosterone (see Figure 17.).

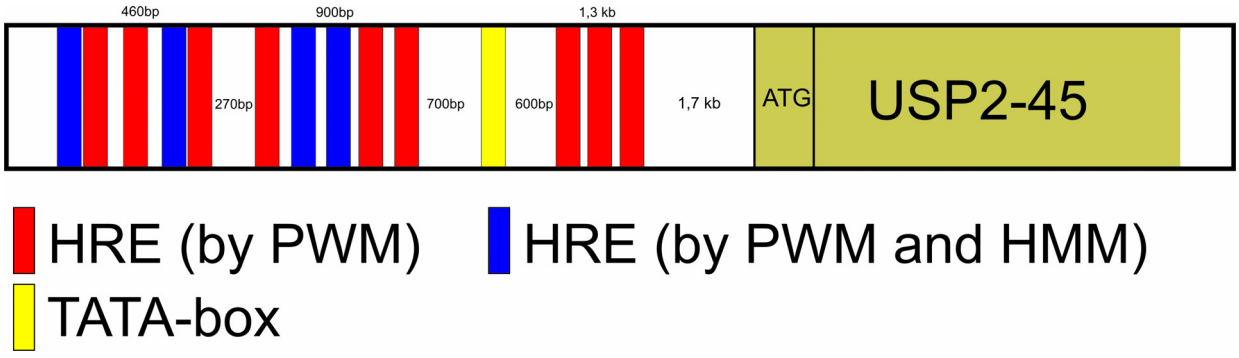

Figure 17. Predicted hormone response elements located in the promoter region of the mouse Usp2 gene. The HRE's predicted only by using the computational statistic models Position Weight Matrix (PWM) are indicated as red segments and the ones predicted by both PWM and the Hidden-Markov-Model (HMM) are indicated as blue segments [274].

An induction by aldosterone of the shorter Usp2-45 isoform, but not of the Usp2-69 isoform, was also detected in mpkCCDcells (see below).

To test whether the aldosterone-induced Usp2-45 mRNA induction translates in vivo into an increase in Usp2-45 protein, we performed Western blotting of proteins prepared from microselected segments of connecting tubule and cortical collecting duct (CNT/CCD) obtained from mice subjected to the same hormonal treatment as previously performed for the mRNA studies.

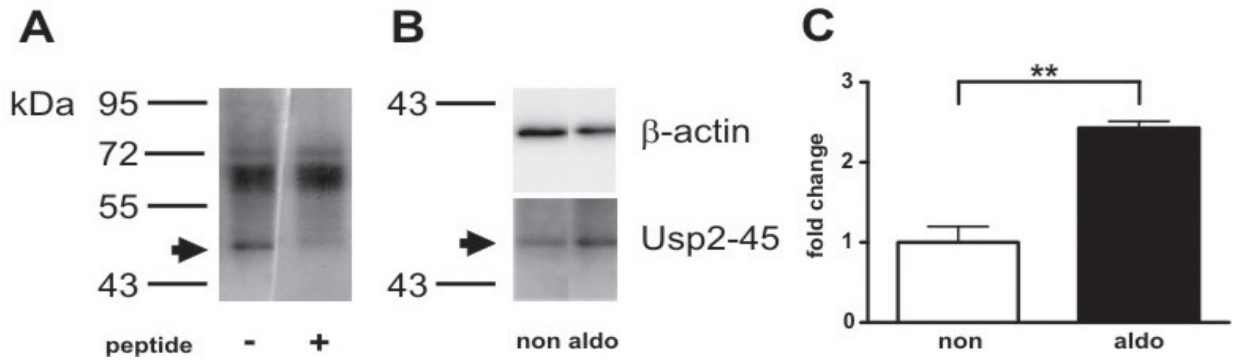

Figure 18. Usp2-45 detected by Western blot in connecting tubule and cortical collecting duct (CNT/CCD). Aldosterone treatment and tubule microselection were as described for mRNA preparations. (**A**) Western blot with rabbit anti-mouse Usp2-45 antibody ± immunizing peptide identifies a 45 kD band that is competed by immunizing peptide. (**B**) Representative pair of Western blots from a control and an aldosterone treated mouse. The upper panels show the actin band and the lower panels the Usp2-45 band visualized on the same blots. (**C**) The Usp2-45 signals were normalized to the actin signal of the same animal (n = 3 for each condition) and the means are shown. **P<0.01.

As shown in Figure 18., the polyclonal rabbit anti-mouse Usp2-45 antibody recognized a 45 kD band that was blocked by competing immunizing peptide. This band was selectively increased 1 hour after an aldosterone injection by a factor of approximately 2, as previously shown for the mRNA.

The fact that the Usp2-45 mRNA indeed encodes an active ubiquitin-specific protease was confirmed by the fact that its core domain produced in *E. coli* very efficiently cleaves ubiquitin off from a test substrate in vitro (Oberfeld, Verrey and Pos, unpublished results and [285]).

To test the possible impact of Usp2-45 on ENaC function, we co-expressed it with ENaC in *Xenopus laevis* oocytes and compared the effect of Usp2-45 to that of Sgk1, the kinase that inhibits endogenous Nedd4-2. Co-expression of Usp2-45 with ENaC increased the level of amiloride-sensitive Na^+ current nearly 4-fold, an effect similar to that of Sgk1 (Figure 19.A, Figure 20.A and B). Interestingly, co-expression of Sgk1 with Usp2-45 did not further increase ENaC function, suggesting that these two enzymes have an impact on the same regulatory mechanism (Figure 19.A and Figure 20.B).

To test whether the effect of Usp2-45 was mediated by its protease activity, we mutated its cysteine 67 that corresponds to one of the catalytically active amino acids of its cysteine protease motif [284, 286]. Consistent with a requirement for an enzymatically active Usp2-45, this mutant did not affect ENaC activity at the membrane (Figure 19.B and Figure 20.C). To control whether this effect of Usp2-45 was specific for this ubiquitin-specific protease, we carried out the same experiment with another randomly chosen ubiquitin-specific protease, Usp15. The results indicated that the effect of Usp2-45 is specific, since co-expression of Usp15 in contrast rather decreased ENaC function (Figure 19.C and Figure 20.D). It is probable that this latter effect is due to the deubiquitylation of other proteins that interfere with the expression of ENaC.

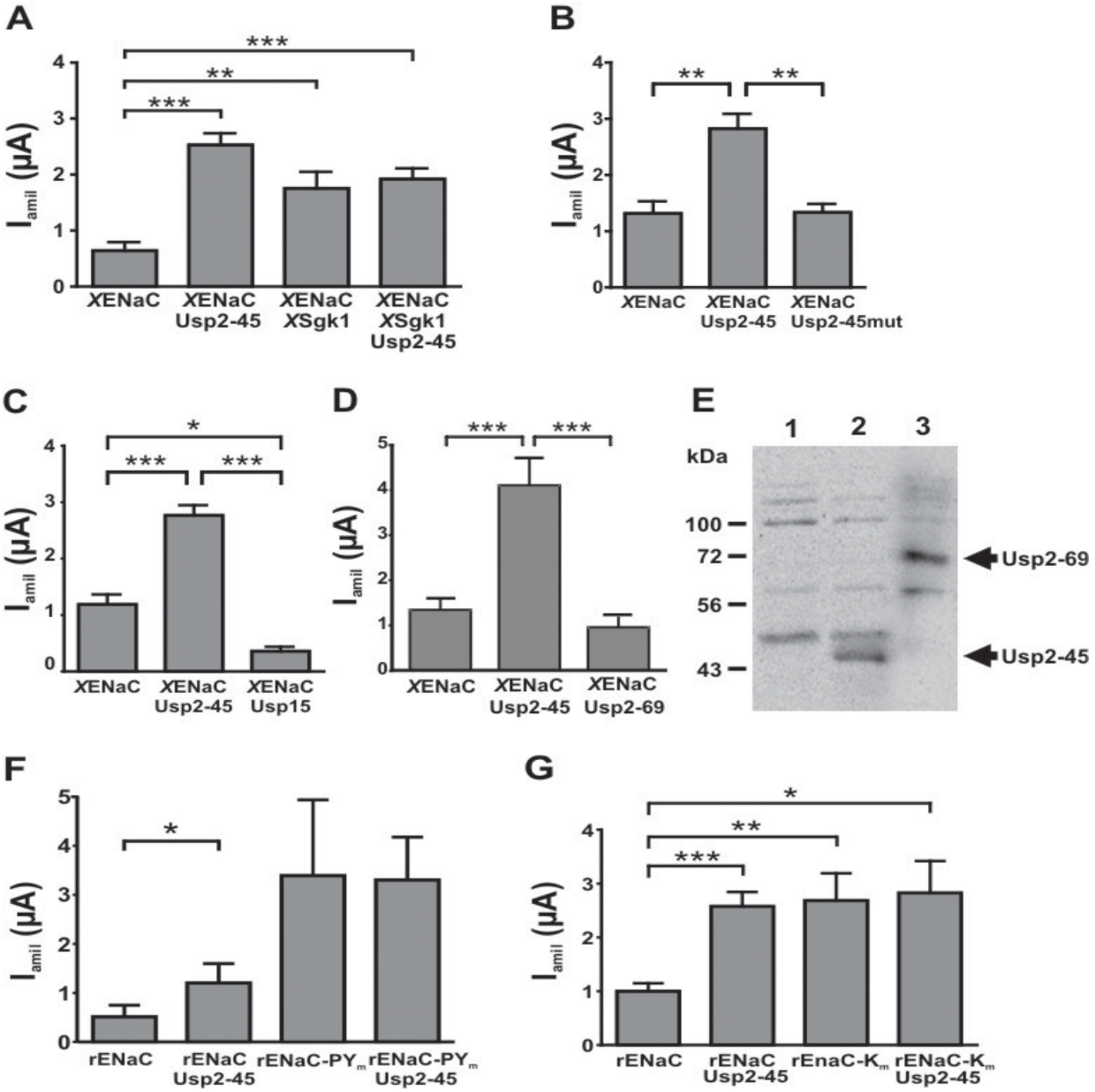

Figure 19. Usp2-45 increases amiloride-sensitive Na^+ current (I_{amil}) carried by coexpressed $\alpha\beta\gamma$ENaC. Original tracings are shown in Figure 20. of the Supporting Material. (**A**) Summary of amiloride-sensitive whole-cell currents at a holding potential of -100 mV. Coexpression of Usp2-45 or of *X*Sgk1 increased the ENaC-mediated amiloride-sensitive I_{amil} to a similar extent. These effects were not additive. Means ± SEM, n = 23 - 27. **P<0.01, ***P<0.001. (**B**), (**C**) and (**D**) Characterization of Usp2-45 stimulatory effect on I_{amil} in Xenopus oocytes. (**B**) Cys67Ala protease dead Usp2-45 mutant (Usp2-45mut) does not stimulate XENaC current, I_{amil} at -100 mV. Means ± SEM, n = 12 - 15. **P<0.01. (**C**) Another Usp (Usp15) does not stimulate XENaC-mediated I_{amil} at -100 mV. Means ± SEM, n = 20 - 24, *P<0.05, ***P<0.001. (**D**) The Usp2-69 isoform does not stimulate XENaC-mediated I_{amil} at -100 mV. Means ± SEM, n = 20 - 22. ***P<0.001. (**E**) Western blot made with lysate of control- and Usp2-45 and Usp2-69 expressing Xenopus oocytes and incubated with a pan-Usp2 antibody shows specific bands corresponding to each isoform, confirming that both were similarly expressed and that the lack of ENaC stimulation by Usp2-69 was not due to a lack of expression of this isoform. (**F**) Effect of Usp2-45 on ENaC-mediated I_{amil} is prevented by mutation of rENaC PY motif (rENaC-PY_m). I_{amil} at -100 mV is shown. Means ± SEM, n = 4 independent experiments with a total of 20 oocytes, *P<0.05 using paired t-test. (**G**) Effect of Usp2-45 on ENaC-mediated I_{amil} is prevented by mutation of rENaC ubiquitylation site (lysine-mutant rENaC-K_m). I_{amil} at -100 mV is shown. Means ± SEM, n = 4 - 5 independent experiments with a total of 20 and 25 oocytes, respectively, *P<0.05, **P<0.01, ***P<0.001.

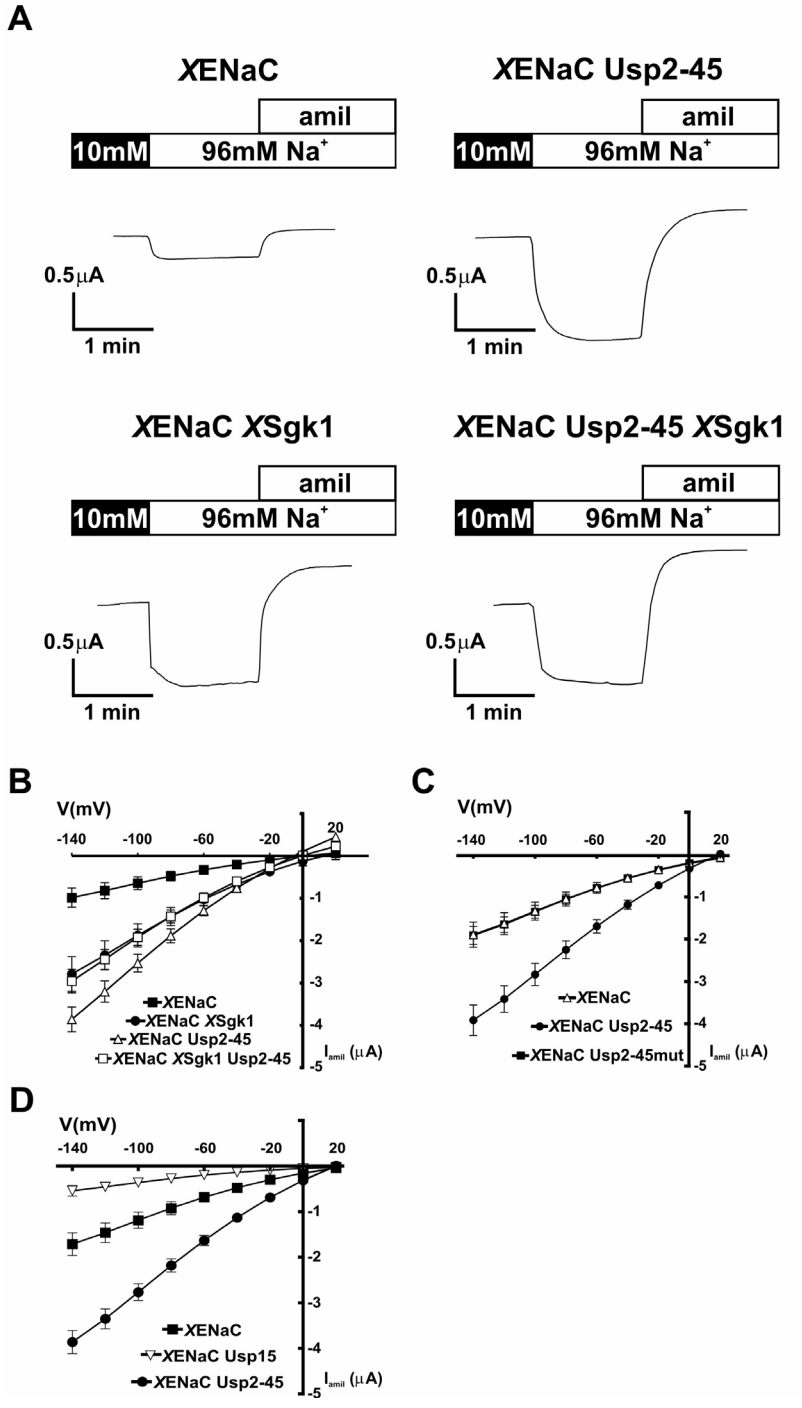

Figure 20. Usp2-45 increases I_{amil} carried by coexpressed αβγ*X*ENaC. (**A**) Typical recordings of *X*ENaC-mediated amiloride-sensitive Na^+ current (I_{amil}) with or without coexpression of *X*Sgk1 and/or Usp2-45 in Xenopus laevis oocytes clamped at -50 mV. Bath sodium concentrations and the presence of 10 μM amiloride are indicated. Switching from 10 to 96 mM Na^+ increased inward current that was blocked with 10 μM amiloride. (**B**) Current-voltage (I-V) relationships of the amiloride-sensitive current (n = 23 - 27 oocytes from 4 - 5 batches). (**C**) and (**D**) Characterization of Usp2-45 stimulatory effect on I_{amil} in Xenopus oocytes. (**C**) Cys67Ala protease dead Usp2-45 mutant (Usp2-45mut) does not stimulate *X*ENaC current, current-voltage (I-V) relationship (n = 12 – 15). (**D**) Another Usp (Usp15) does not stimulate *X*ENaC-mediated I_{amil}. Current-voltage (I-V) relationship, (n = 20 – 24).

Additionally, we also coexpressed the longer Usp2-69 isoform that turned out not to impact onto ENaC function (Figure 19.D). In this case, we also verified by Western blotting that this ubiquitin-specific protease isoform was indeed expressed as expected (Figure 19.E).

We then asked whether Usp2-45 interferes with Nedd4-2-dependant regulation of ENaC, a mechanism that involves the interaction of Nedd4-2 with PY-motifs present on the C-termini of the ENaC subunits and is defective in Liddle's syndrome [230]. ENaC devoid of PY-motifs (alpha Y673A, beta Y618H, gamma Y628A) is resistant to Nedd4-2 and thus displayed a higher level of amiloride-sensitive Na^+ current than wild type ENaC. This current was indeed resistant to the stimulatory action of Usp2-45 (Figure 19.F). This observation supports the hypothesis that Usp2-45 cleaves off ubiquitin moieties that Nedd4-2 ligates onto ENaC (see scheme in Figure 24.). The involvement of direct ENaC ubiquitylation was then verified by coexpressing Usp2-45 with ENaC devoid of potential ubiquitylation sites (lysine residues of the intracellular NH_2-terminal domains of all three subunits mutated to arginine) in *Xenopus* oocytes. As expected for the case that Usp2-45 deubiquitylates ENaC directly, this mutant channel was not activated in the presence of Usp2-45 (Figure 19.G).

To verify that the effect of Usp2-45 on ENaC function was not an artefact due to the coexpression of two proteins in *Xenopus* oocytes but also takes place with endogenous ENaC in CCD cells, we addressed this question using the mouse cortical collecting duct cell line mpkCCD_{c14} [278]. We actually first verified whether Usp2-45 is expressed and regulated by aldosterone in these cells. The relative expression of the two isoforms in mpkCCD_{c14} cells was similar to the observation made in microdissected CNT/CCD with a 10-fold higher expression of the Usp2-45

mRNA. The time course of the regulation by aldosterone revealed that only the Usp2-45 isoform was regulated (Figure 21.A).

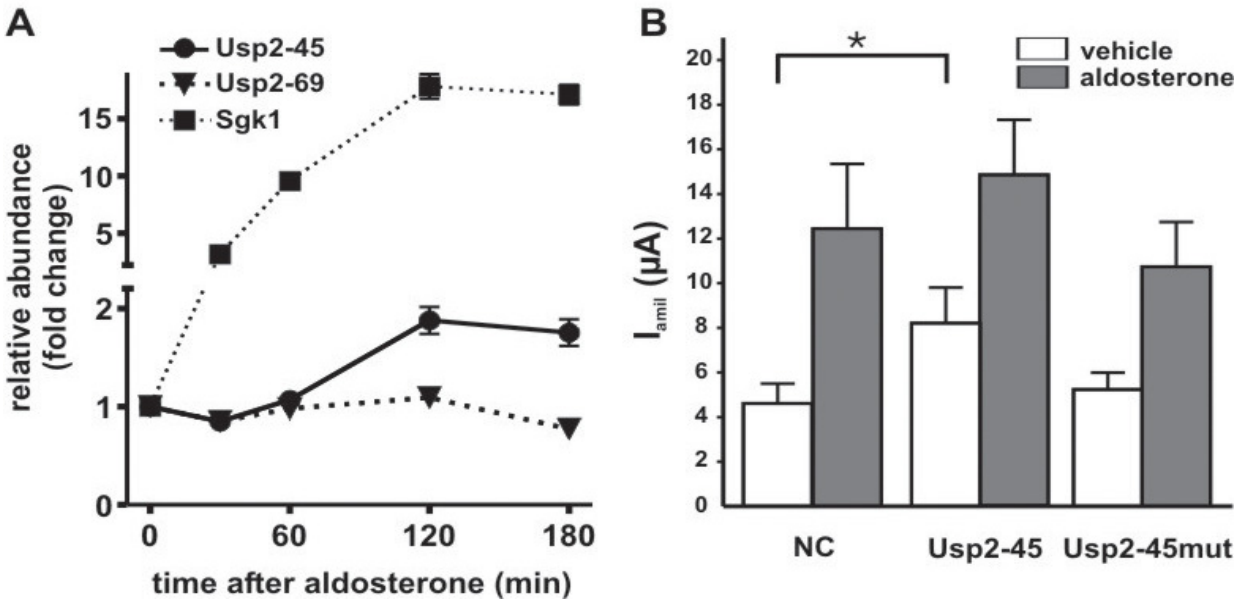

Figure 21. Expression of endogenous and exogenous Usp2-45 in mpkCCDcl4 cells. (A) Regulation of endogenous Usp2-45, Usp2-69 and Sgk1 mRNAs by aldosterone (10^{-6} M, see Methods in Supporting Materials and [279]) in $mpkCCD_{c14}$ cells. Usp2-45 upregulation is slightly more delayed than that of Sgk1. Unlike its isoform Usp2-45, Usp2-69 is not induced by aldosterone. Baseline mRNA abundance of Usp2-45 was 10-fold higher than that of Usp2-69. Fold changes of normalized means relative to β-actin ± SEM are given, n = 3. The isoform-specific Taqman® real time PCR primers and probes are shown in supporting information. (**B**) Baseline transepithelial I_{amil} across $mpkCCD_{cl4}$ cell monolayers cultured on permeable supports is significantly increased in cells overexpressing Usp2-45 compared to vector-transduced negative control cells (NC) and cells transduced with protease-dead Usp2-45mut (open bars) (*$P < 0.05$, n = 9-12). Aldosterone (300 nM) given for 3 hours increased I_{amil} similarly in the monolayers made of the different $mpkCCD_{cl4}$ cell populations (grey bars).

To investigate the effect of Usp2-45 on the transepithelial Na^+ transport function, we expressed exogenous Usp2-45 as well as mutant protease-dead Usp2-45 in mpkCCD cells by retroviral transduction and verified by Western blot that the expression levels were equal (Figure 22.).

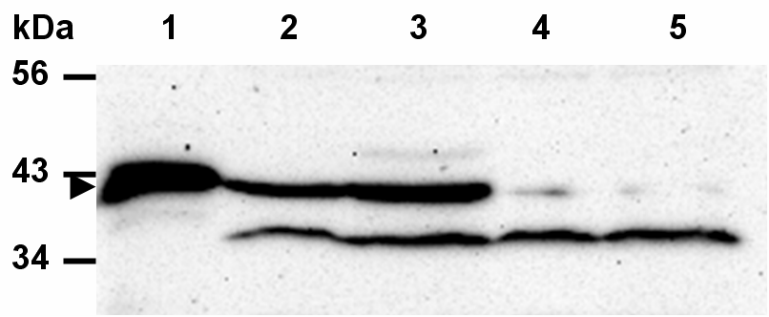

Figure 22. Expression of endogenous and exogenous Usp2-45 in $mpkCCD_{cl4}$ cells. Western blotting of Usp2-45 in $mpkCCD_{c14}$ cells. In transduced $mpkCCD_{c14}$ cells, Usp2-45 wild type (lane 2) and protease dead mutant (lane 3) appear as ~41 kDa bands (arrow head) of similar intensity. Endogenous Usp2-45 is not clearly visible in wild type and vector-transduced $mpkCCD_{cl4}$ cells (lane 4 and 5). Lane 1 shows Usp2-45 expressed in Xenopus laevis oocytes. The band at ~36 kDa in lane 2 - 5 ($mpkCCD_{c14}$ lysate) is unspecific.

As expected if ENaC expression and activity was limited by its ubiquitylation, expression of functional Usp2-45 increased the baseline transepithelial amiloride-sensitive Na^+ transport (Figure 21.B). In contrast, the expression of the protease-dead mutant had no effect. The increase in Na^+ transport induced by aldosterone (300 nM, see Materials and Methods and [279]) given for three hours was not modified significantly by the expression of wild type and mutant Usp2-45, as expected considering that short-term aldosterone prevents the Nedd4-2-mediated ubiquitylation of ENaC by inducing SGK1. These results obtained in CCD cells confirm that Usp2-45, an ubiquitin-specific protease identified because of its in vivo regulation by aldosterone, also acts on endogenous ENaC in kidney tubule cells.

To directly test the hypothesis that Usp2-45 counteracts the effect of Nedd4-2 by deubiquitylating ENaC, we used Hek293 cells as expression system.

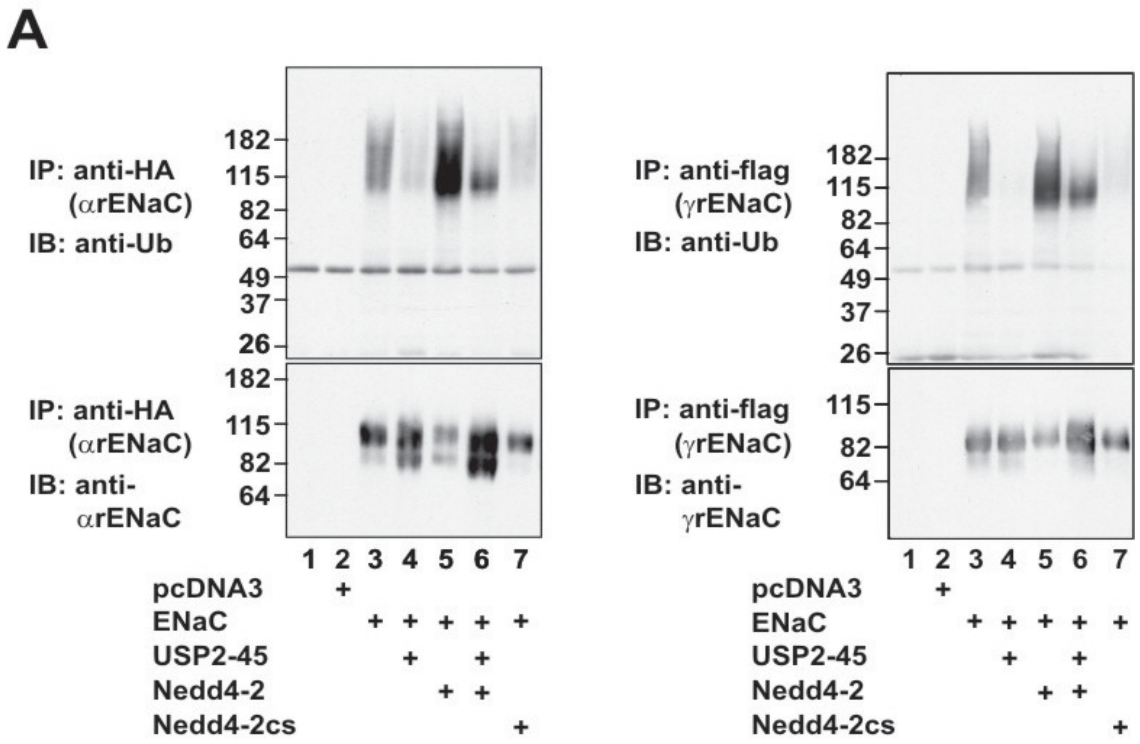

Figure 23. Inverse regulation of ENaC ubiquitylation and ENaC amount. (**A**) The ubiquitylated and the total amount of ENaC expressed in Hek293 cells are visualized by Western blotting (IB) of immunoprecipitated (IP) HA- and Flag-tagged α- and γrENaC subunits, respectively. The MW of marker proteins is indicated in kDa. Co-expression of Nedd4-2, and not of ligase-inactive Nedd4-2 (Nedd4-2cs), increases the ubiquitylation of both tested ENaC subunits, whereas it decreases their total amount. In the presence of Usp2-45, ENaC ubiquitylation is decreased and the total subunit amount increased.

Coexpression of Nedd4-2 with ENaC in Hek293 cells massively increased the ubiquitylated form of the channel that was visualized with anti-ubiquitin antibody on immunoprecipitates of α- and γ–ENaC, thus confirming that ENaC was itself ubiquitylated (Figure 23.A). The impact of ubiquitylation on the total amount of ENaC was demonstrated by the decreased amount of ENaC visualized in the same samples with anti-ENaC antibody. Importantly, Usp2-45 co-expression decreased the amount of ubiquitylated ENaC and increased total ENaC,

demonstrating that Usp2-45 antagonizes the effect of Nedd4-2 by directly deubiquitylating ENaC.

The low level of ubiquitylated ENaC and the substantial level of total ENaC observed with Usp2-45 co-expression were similar to those observed with Nedd4-2cs, a catalytically inactive Nedd4-2 (Figure 23.A).

Discussion

1. Early Aldosterone-Regulated Gene Products

The number of mRNAs the level of which was changed ≥ 2-fold in mouse ASDN within one hour after an aldosterone injection is relatively low (22 mRNAs). It is noteworthy that the signal intensity of these regulated mRNAs on control mice arrays differs by more than two orders of magnitude indicating large differences in their absolute baseline expression levels (see Figure 16.A). Not unexpectedly, the gene product with the highest absolute expression level and the second highest relative level of induction is Sgk1, the only early aldosterone-regulated gene product of mammalian kidney that has been identified earlier and shown to activate ENaC.

The gene product with the highest fractional increase (7.2-fold in Affymetrix screen, 5.6-fold by real-time RT-PCR) is Grem2 (PRDC), an antagonist of bone morphogenic proteins (BMP) 2, 4, 6 and 7 [287]. BMP's are the largest group within the TGFβ superfamily and regulate gene transcription via heteromeric BMP receptors and Smad pathways [288, 289]. Thus aldosterone-induced Grem2 might support cellular differentiation by preventing BMP-mediated transcriptional activation of genes as for instance that of the inhibitor of differentiation 1 (Id1) [290].

The second upregulated gene product on the list is the already mentioned Sgk1 that is followed by two members of the CREB family of transcription factors, activating transcription factor 3 (Atf3) and cAMP responsive element modulator (Crem). Atf3 is actually a transcription inhibitor that is known to be induced by

stress and to exert its inhibitory function on certain promoters in cooperation with Smad3. Such an inhibitory effect has been demonstrated in epithelial cell lines on the gene of Id1, the inhibitor of differentiation mentioned above as a putative target of Grem2-mediated inhibition [290]. Thus it appears that two of the most highly induced early aldosterone-regulated gene products (Grem2 and Atf3) act onto common targets via distinct pathways.

Besides hypothetical effects as the one suggested above for Atf3, the actual role in aldosterone-sensitive distal nephron of the two early aldosterone-induced CREB family members Atf3 and of Crem remains to be established. In this context, it is noteworthy that aldosterone synergizes with vasopressin and with other cAMP-inducing hormones in stimulating Na^+ and water reabsorption in these kidney tubular segments [291]. This synergy might implicate also transcriptional effects, besides posttranscriptional regulatory mechanisms at the level of ENaC and AQP2 surface expression and function, since AVP has been shown to regulate many genes in cultured collecting duct cells [292].

Our list of early aldosterone-regulated mRNAs included also 13 mRNAs that are downregulated (Table 2). The known mRNA with the highest fractional decrease (0.3-fold in Affymetrix screen, 0.2-fold by real-time RT-PCR) encodes G0s2 a putative G0/G1 switch gene the downregulation of which might prevent proliferation and thus favor differentiation. The function of this gene product might be to stimulate cell proliferation and thus aldosterone would favour cellular differentiation by decreasing its expression [293].

Interestingly, there are also two transport proteins among the downregulated gene products, namely the polyspecific organic anion transporter Oat3 (Slc22a8) and the colonic H^+,K^+-ATPase.

The next downregulated gene product on the list is Oat3 (Slc22a8), a polyspecific organic anion transporter expressed in many tissues, including kidney proximal tubule and adrenal cortex. One of its functions that might be relevant in the context of aldosterone action is its capacity to transport steroids, for instance cortisol, across the plasma membrane [294]. It is also interesting that the colonic H^+,K^+-ATPase, which participates in K^+ absorption and H^+ secretion from the tubular lumen, appeared among the downregulated gene products. This supports the notion that the stimulation of K^+ secretion by aldosterone is not only a by-product of the increase in Na^+ reabsorption but also a distinct action of aldosterone involving different gene products. Indeed, a decrease in apical H^+,K^+-ATPase expression (and function) is expected to lead to a decrease in K^+ (re)absorption from the tubular lumen.

2. Role of Deubiquitylation in Aldosterone Action

The regulation of the gene products discussed in the previous section appears to favour a decrease in proliferation of aldosterone target cells and to foster their differentiation. Additionally our screen identified with Sgk1 and Usp2-45 two aldosterone-induced gene products interfering with protein ubiquitylation. As mentioned in the Introduction, Sgk1 was identified earlier as aldosterone-induced mRNA and protein and its effect on ENaC function has been shown to be mediated to a large extent by its inhibitory action on the ubiquitylating enzyme Nedd4-2.

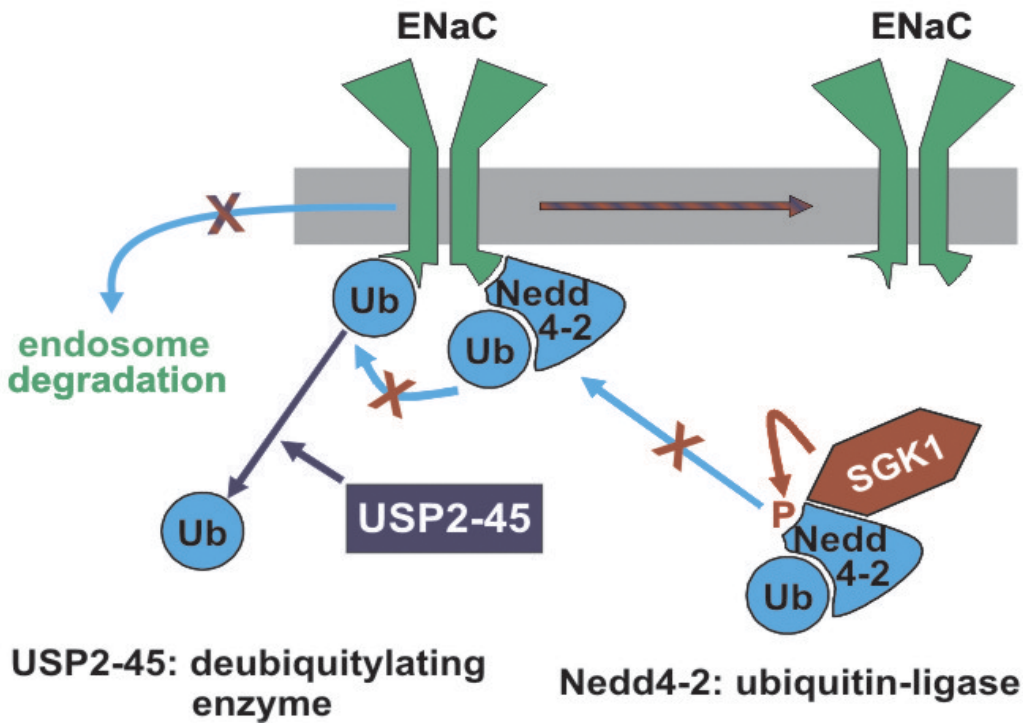

Figure 24. Schematic representation of the convergent effect of Sgk1 and Usp2-45 that both decrease the amount of ubiquitylated ENaC and thus increase the amount of intact channel. The subcellular localization of these ubiquitylation/ deubiquitylation reactions has not yet been identified.

We have now indeed shown that the newly identified aldosterone-induced Usp2-45 directly deubiquitylates ENaC in the Hek273 cell expression system (Figure 23.A). As expected if the ubiquitylation of ENaC would lead to ENaC degradation, the presence of Usp2-45 not only decreases the amount of ubiquitylated ENaC but also increases the total amount of ENaC. This biochemical evidence of ENaC deubiquitylation by Usp2-45 is supported by functional experiments made in *Xenopus* oocytes that show an increase in ENaC function specifically mediated by Usp2-45 and that depends on the integrity of its protease motif. Furthermore, we show both that the disruption of ENaC PY motifs and the disruption of ENaC lysine residues potentially targeted by Usp2-45 prevent the action of Usp2-45 on ENaC function. These results also strongly support the hypothesis that Usp2-45 acts on ubiquitin moieties ligated onto ENaC by the ubiquitin ligase Nedd4-2.

The fact that a deubiquitylating enzyme acting on ENaC is induced by aldosterone strengthens the notion that ubiquitylation plays a central role in the short-term regulation of ENaC. Although deubiquitylation of target proteins has been described since the early days of the ubiquitin system discovery [295], it has only recently gained considerable interest as a regulatory mechanism (for a review on deubiquitylation see [283]). The mouse as the human genome encodes approximately 100 putative deubiquitylating enzymes, the function of most of which is not known.

Probe Set ID	Gene	Symbol
1451080_at	ubiquitin specific peptdiase 1	Usp1
1417169_at	**ubiquitin specific peptidase 2**	**Usp2**
1417168_a_at		
1425022_at	ubiquitin specific peptidase 3	Usp3
1425023_at		
1450892_a_at	ubiquitin specific peptidase 4	Usp4
1419920_s_at	ubiquitin specific peptidase 7	Usp7
1422883_at	ubiquitin specific peptidase 8	Usp8
1420922_at	ubiquitin specific peptidase 9	Usp9
1428193_at		
1428194_at		
1450039_at		
1448230_at	ubiquitin specific peptidase 10	Usp10
1434483_at	ubiquitin specific peptidase 12	Usp12
1416208_at	ubiquitin specific peptidase 14	Usp14
1437714_x_at		
1431951_a_at	ubiquitin specific peptidase 16	Usp16
1418191_at	ubiquitin specific peptidase 18	Usp18
1424656_s_at	ubiquitin specific peptidase 19	Usp19
1455205_a_at		
1423777_at	ubiquitin specific peptidase 20	Usp20
1448939_at	ubiquitin specific peptidase 25	Usp25
1426709_a_at	ubiquitin specific peptidase 33	Usp33
1434392_at	ubiquitin specific peptidase 34	Usp34
1434393_at		
1455237_at	ubiquitin specific peptidase 36	Usp36
1428592_s_at	ubiquitin specific peptidase 38	Usp38
1437007_x_at	ubiquitin specific peptidase 39	Usp39
1460209_at		
1424866_at	ubiquitin specific peptidase 43	Usp43
1427990_at	ubiquitin specific peptidase 45	Usp45
1427991_s_at		
1426976_at	ubiquitin specific peptidase 47	Usp47
1426977_at		
1419277_at	ubiquitin specific peptidase 48	Usp48
1419278_at		
1424056_at		
1426700_a_at	ubiquitin specific peptidase 52	Usp52
1452385_at	ubiquitin specific peptidase 53	Usp53

Table 3. Present called Usp‘s in either the aldosterone or control condition in mouse CNT/CCD.

Affymetrix© probe set IDs, gene name and gene symbol are listed

The Affymetrix screen presented in this work revealed that approximately 26 of these Usp’s are expressed (Table 3.) in the aldosterone target cells of the mouse kidney tubule (present calls), but only Usp2-45 appeared in the list of regulated RNAs (Table 2.). Based on our results, the deubiquitylation of ENaC by Usp2-45 appears to be quite specific for Usp2-45 since none of the four other Usp’s that we

tested in expression systems activated and/or deubiquitylated this channel (Figure 19.C, D and unpublished results). As yet, actually only few examples of plasma membrane protein deubiquitylation have been published. One such example is the deubiquitylation of yeast membrane permeases that leads to their targeting to the vacuole (equivalent to the mammalian lysosome) by the ubiquitin-specific protease Doa4 [296]. Another is the deubiquitylation and stabilization of the epidermal growth factor receptor by the endosomal ubiquitin isopeptidases AMSH and UBPY [297, 298].

The identification of ENaC as target of Usp2-45 represents the first report of a hormonally controlled channel-deubiquitylating mechanism. It is noteworthy to mention that this effect is not selective for kidney tubule, since Usp2-45 is also expressed and regulated in distal colon, yet another target epithelium of aldosterone (Fakitsas and Verrey, unpublished results). Previously published data on Usp2 suggests that its isoform Usp2-69 is regulated by androgens in liver and prostate where it deubiquitylates and stabilizes fatty acid synthase [286]. Furthermore, Usp2 was shown to be regulated in bone by parathyroid hormone [299], indicating that the *Usp2* gene products mediate various important hormonally regulated functions.

The fact that the stimulatory action of Usp2-45 on ENaC depends on prior ENaC ubiquitylation indicates however that Usp2-45 induction cannot explain the effect of aldosterone on Liddle-type mutant ENaC (see Introduction [300, 301]). Thus it is expected that still other aldosterone-induced gene products, possibly also identified in our screen, act via different pathways on ENaC expression and function.

In summary, our list of early aldosterone-regulated gene products contains two induced elements that interfere with the ubiquitylation of ENaC. Besides Sgk1 we identified Usp2-45 that we show to deubiquitylate ENaC in expression systems and thus represent a first example of a deubiquitylating enzyme acting on a channel.

2.1. Outlook

The results presented in this PhD thesis have contributed to the understanding of the physiologically relevant early aldosterone-mediated effects on Na^+ absorption in the distal nephron. Using a novel approach involving Affymetrix® GeneChips® we identified new early aldosterone targets. Previously only two gene products, K-Ras2 and Sgk1, had been identified and experimentally shown to have an impact on ENaC function. In addition we now add Usp2-45 as a new player acting on the same axis of ENaC-mediated aldosterone effects. Moreover we characterized the role of Usp2-45 as the first hormonally regulated deubiquitylating enzyme known to be acting on a channel protein.

This study has had and will continue to have an impact on future studies focusing on different and more tissue specific roles of Usp2-45 e.g. in heart or colon. These continuing studies range from solving the crystal structure (the main focus of Benjamin Oberfeld's project) to the possible effects of Usp2-45 on the voltage-dependent Na^+ channel in heart, which is the focus of studies at the Lab of Hugh Abriel in Lausanne.

The microarray studies provided a valuable initial list of candidate genes. For example, four interesting genes from this list, Hv1, Usp53, Atf3 or Grem2, which are all significantly differentially expressed in the aldosterone-treated group, can be further functionally characterized for example, through the use of inducible exogeneous expression in cell model systems. Another potential strategy is the tissue specific knock-out of the genes in order to investigate the resulting phenotype. These studies, which are now the focus of Gabriele Adam's and Tina Dauwalder's projects, might give deeper insights into aldosterone-dependent

proton transport as well as reveal the transcriptional switches involved in the aldosterone-ENaC cascade.

A second goal of our work, which was to determine whether Usp2-45 has regulatory effects on target(s) in addition to kidney, was approached through a second set of microarray studies performed on heart and colon tissue dissected from mice previously used for the kidney microarray experiments (see Results section). This study indicated a role for transcriptional factors like NF-kappa, supporting the idea that in kidney and colon aldosterone mediates cellular differentiation rather than proliferation.

In summary, the gene lists from these microarray studies have both shed light on the physiological mechanism of early aldosterone action and allowed us to pose new questions regarding the complex picture of aldosterone related Na^+ absorption.

Addendum

Differential Gene Regulation by Aldosterone in Target Tissues

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[#] PF and UW contributed equally to this work

1. Introduction

The systemic actions of aldosterone are well documented; however, in comparison, our understanding of the cellular and molecular mechanisms by which aldosterone orchestrates these actions is rudimentary. Aldosterone is thought to exert most of its physiological actions by modifying gene expression. It is now apparent that aldosterone represses more genes (~ 2/3) as it induces. Several aldosterone-sensitive genes, including Sgk1, Usp2-45 and small, monomeric Kirsten Ras GTP-binding protein (Ki-ras) have been identified. The cis-acting elements and some molecular mechanisms bestowing corticosteroid

sensitivity onto these and many other genes have been described to some extent but the cell-specificity conferring mechanism of this action remain to a large extent to be unravelled. Induction of Ki-Ras and Sgk1 has been shown to be necessary and sufficient to elicit some portion of aldosterone action in epithelia. These two signaling factors are components of a converging regulatory network with phosphatidylinositol 3-kinase positioned between them that enables both stabilizing the epithelial Na+ channel (ENaC) in the open state as well as increasing the number of ENaC in the apical membrane. This aldosterone-regulated signalling network contains many potential sites for feedback regulation and cross-talk from other cascades and potentially impinges directly also on cellular differentiation [78].

Besides acting on epithelial target-cells of kidney, colon, sweat glands etc., aldosterone acts also on non-epithelial sites like heart, brain and vasculature [302].

Moreover, numerous experimental and clinical studies indicate that aldosterone participates in cardiac alterations associated with hypertension, heart failure, diabetes and other pathological entities. It is important to mention that dietary salt is a key factor in aldosterone-mediated cardiovascular damage, since damage was more evident in animals on a high-salt diet than on a low salt diet. Some pathophysiological actions of this hyperaldosteronism in cooperation with salt excess involve development of extracellular matrix and fibrosis, inflammation, stimulation of reactive oxygen species production, endothelial dysfunction, cell growth and proliferation. Many studies have found that local extra-adrenal production of aldosterone in brain blood vessels and in the heart, contributes to the pathological actions of this mineralocorticoid [303].

1.1. Mechanisms of Aldosterone Action

Inhibitors of transcription and translation, as well as other experimental manoeuvres, have been employed to demonstrate that induction of gene expression is a necessary mechanism for the full action of aldosterone in epithelia (reviewed in Refs. [19, 304-306]). However, the possibility, that gene repression is also needed for aldosterone action, has been more difficult to demonstrate. This is in part due to the fact that genes negatively regulated by aldosterone have not been well described. Recently, a number of aldosterone-repressed transcripts have been identified [42, 292]. It is now apparent that aldosterone represses approximately as many genes as it induces. It follows that aldosterone-induced gene repression plays an important, but yet undefined role in the cellular response.

In this study we compare aldosterone-sensitive regulatory gene products from three tissue types (kidney, colon and heart) that were affected by the short-term treatment of mice with aldosterone.

2. Materials and Methods

2.1. RNA purification, Microarray Sample Preparation, Hybridization and Scanning

The organs for this study were harvested from the same female C57BL/6JOlaHsd mice (Harlan), used in Fakitsas et al. [307]. The hearts were removed and the colon mucosas (up to 2 cm upstream of the rectum) were carefully scraped off using a sharp blade. Tissues were directly transferred into RLT lysis buffer (Qiagen RNeasy Mini Kit, Qiagen) for total RNA isolation.

The quantity and quality of the isolated RNA was determined using a NanoDrop® UV-spectrophotometer ND 1000 (NanoDrop Technologies) and NanoChip® for a Bioanalyzer 2100 (Agilent Technologies). Only those samples with a 260 nm/280 nm ratio between 1.8–2.1, a 28S/18S ribosomal RNA ratio within 1.5–2 and a RNA Integrity Number (RIN; Agilent Technologies) >7 (range 1-10 with 10 for best RNA quality) were further processed for target cRNA preparation. RNA amplification and labelling was performed according to a one-cycle cDNA synthesis protocol. Total RNA (<2 µg) was used for first and second strand cDNA synthesis with the GeneChip® One-Cycle Synthesis Kit (Affymetrix, heart) or the SuperScript cDNA Synthesis Kit (Invitrogen, colon) in the presence of an oligo-dT primer containing a T7 RNA polymerase promoter site. The resulting cDNA was then in vitro transcribed in the presence of biotin-labelled nucleotides (UTP's) using the GeneChip® IVT Labeling Kit (Affymetrix Inc., P/N 900449), purified and quantified using the BioRobot Gene Exp – cRNA Target Prep (Qiagen AG). The labelled

cRNA were tested for quality using the Agilent 2100 Bioanalyzer® (NanoChip®, Agilent Technologies) and for quantity using the ND-1000 NanoDrop® UV-spectrophotometer (NanoDrop Technologies). Only samples showing labelled cRNA average size distribution higher than 0.5 kb were accepted for hybridization. Target cRNA Hybridization and GeneChipGeneChip® Scanning were performed accordingly to manufacturers' manual.

2.2. Quality Control of Microarrays and Statistical Analysis

Four, (in case of colon and heart) or six (in case of CCD/CNT) GeneChips® per condition were used for hybridization. Each hybridization was performed with target RNA from individual mice. Array images were examined visually for artefacts and B2 Oligo performance. Raw data processing was performed using the Affymetrix® Gene Chip Operating System Software (GCOS 1.4, Affymetrix) and the Genespring software 7.2 (Agilent). Summarization for the respective probe sets giving rise to the actual expression values was carried out using the GCRMA algorithm as described by Wu and Irizarry, 2004.

Genes were filtered out from values in Genespring 7.3 when not showing a non-logarithmic expression value of at least 50 in 3 out of 4 and 4 out of 6, respectively, of all replicate measurements of at least one condition. For each tissue, unequal variance t-tests were applied to detect genes that were significantly differentially expressed between aldosteron treated and control mice. A *P*-value of ≤ 0.05 was defined significant. Finally, the resulting significant genes were filtered for fold changes with a cut-off value of ≥ 1.8 between the conditions.

2.3. Real-Time RT-PCR

Samples of 3 μl RNA (out of 60 μl) were used as templates for reverse transcription using TaqMan® Reverse Transcription Kit (Applied Biosystems) in the presence of 2.5 μM random hexamer primers (Applied Biosystems). Real-time PCR was performed using 3.5 μl of the cDNA as template using the TaqMan® Universal PCR master mix (Applied Biosystems). Primers and probes were designed using Primer Express 2.0 software (Applied Biosystems) to result in amplicons of 70-100 bp that span intron-exon boundaries. Each primer pair was first tested on mouse kidney cDNA and resulted in a single product of the expected size (data not shown). Probes (Table 1.) were labelled with reporter dye FAM at the 5' end and the quencher dye TAMRA at the 3' end (Microsynth). Reactions were run in 96-well optical reaction plates using a Prism 7700 cycler (Applied Biosystems). Thermal cycles were set at 95°C (10 min) and the 40 cycles at 95°C (15 sec) and 60°C (1 minute) with auto ramp time. To analyze the data, the threshold was set to 0.06 (value in the linear range of amplification curves). All reactions were run in triplicates and the abundance of the target mRNAs was calculated relative to a reference mRNA (mouse β-actin). Assuming an efficiency value of 2 (fold-increase in input mRNA required to decrease the cycle number by 1), relative expression ratios were calculated as $R = 2(Ct(\beta\text{-actin}) - Ct(test))$, where Ct is the cycle number at the threshold and test stands for the tested mRNAs. Based on this analysis, some samples (<1/3) were excluded from microarray target RNA synthesis because of a relatively low enrichment in α- and βENaC mRNA or, for aldosterone-treated mice, a Sgk1 upregulation <2-fold. Real-time RT-PCR was performed similarly for controlling the regulation of 8 mRNAs considered as highly regulated by microarray.

3. Results

The RNA samples prepared from single mice were tested in terms of quantity, integrity, enrichment; contamination and response to aldosterone (Figure 15.C and D). These measurements revealed that in micro-isolated CNT/CCD segments ENaC mRNA was enriched 15 - 40-fold over total kidney, whereas thick-ascending limb-specific triple cotransporter Nkcc2 mRNA was not increased. Sgk1 mRNA was enriched 3 - 4-fold over the total kidney preparation and specifically further increased 5 - 6-fold by aldosterone. The manipulation of mice slightly increased plasma corticosterone levels (Figure 14.A and B) but not Sgk1 expression (Figure 15.C and D). Relative to kidney, mouse colon showed an inverted profile of ENaC subunit mRNA expression.

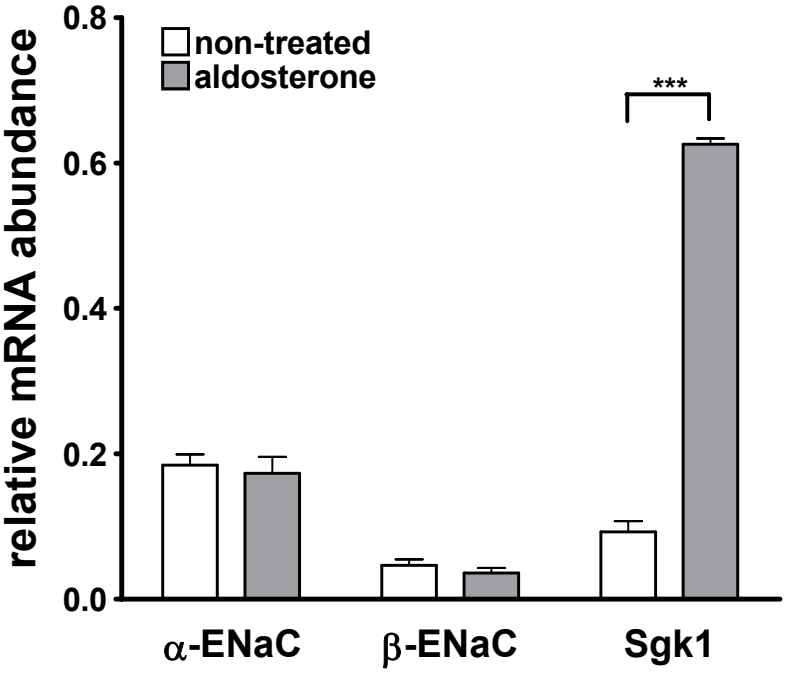

Figure 25. Test for aldosterone stimulation of RNA prepared from colon. Mice were injected with aldosterone and RNA prepared from colon obtained after 1 h. The abundance of α-, βENaC and Sgk1 mRNAs was measured by real-time RT-PCR. Given are means relative to β-actin ± SEM, n = 4 mice. ***P<0.001 by 1 way-ANOVA.

As expected, colon had a higher gene expression of the ENaC α-subunit (5 times higher) than of the β-subunit. Furthermore Sgk1 was around 6.5 fold induced while no induction of ENaC subunits was observed in the aldosterone- versus the non-treated mice (Figure 25).

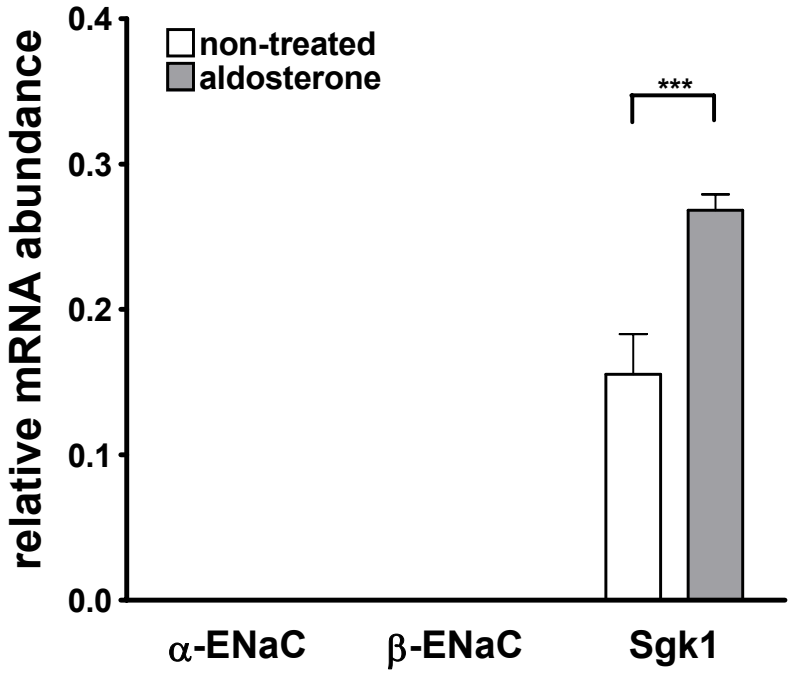

Figure 26. Test for aldosterone stimulation of RNA prepared from heart. Mice were injected with aldosterone and RNA prepared from heart after 1 h. The abundance of α-, βENaC and Sgk1 mRNAs was measured by real-time RT-PCR. Given are means relative to β-actin ± SEM, n = 4 mice. ***P<0.001 by 1 way-ANOVA.

Heart showed very low levels of α- to 'no' levels of β-ENaC expression and no significant regulation of α- or β-ENaC after aldosterone stimulation. A small but significant induction of Sgk1 (around 2 fold) was detected (Figure 26). It must be conceded that this effect might be not only MR but also GR mediated (2-fold induced corticosterone after 1 h of aldosterone injection; Figure 14.A).

More than two thirds of the CNT/CCD-prepared RNA samples fulfilled the selection criteria for quantity, integrity, enrichment, Sgk1 induction and lack of contamination and could be used as template for cDNA amplification and labelled target RNA synthesis. Target cRNAs made from 6 control and from 6 aldosterone-injected mice were separately hybridized to mouse full genome GeneChip® microarrays (MOE 430 v2, Affymetrix). For colon and heart 4 samples per condition were prepared for target cRNA sample preparation and microarray hybridization. Raw array data were processed using the GCRMA algorithms. The data were filtered by setting a cut-off for raw values of RAW ≥ 50 in 4 out of 6 arrays per condition for the case of CCD/CNT. In the case of colon and heart the raw values were filtered by RAW ≥ 50 in 3 out of 4 arrays per condition. Finally a *t*-test was applied with a cut-off *P*-value of $P \leq 0.05$ and using a fold-change limit of 1.8. Genes which passed these criteria were later used to generate an overlap list (Table 4.).

Table 4. Commonly differentially expressed genes in CNT/CCD; colon and heart

gene name	fold change			Gene Bank	Symbol
	CCD/CNT	Colon	Heart		
serum/glucocorticoid regulated kinase	4,5	5,0	n.a.	NM_011361	Sgk1
ubiquitin specific peptidase 2	2,3	2,0	n.a.	AI553394	Usp2
period homolog 1 (Drosophila)	n.a.	2,2	2,4	AF022992	Per1
DNA segment, Chr 17, human D6S56E 5	2,4	n.a.	0,5	NM_033075	D17H6S56E-5

The output list (Table 4.) shows that only 4 genes (Sgk1, Usp2, period homolog1 and DNA segment Chr 17) were commonly differentially expressed with a fold change of ≥1.8 in at least 2 tissues:.Using these criteria, in kidney and colon the only gene products that show differential gene expression are Sgk1 and Usp2.

In order to investigate these data, we focused in detail on the organs colon and

heart. In collaboration with PD C. Cohen we then further investigated 2 gene lists (kidney and colon) focusing on the promoter regions upstream of the origin of transcription. The analysis tool `Bibliosphere` was used to investigate microarray data. Bibliosphere extract knowledge about the gene-gene interactions from available literature databases and promoter analysis of a list of genes. In our case this list of genes is constituted by the lists of significantly expressed genes. The Bibliosphere tool generates figures that show regulatory networks.

In kidney, out of the top 20 induced genes eight are transcription factors (TF). Out of the 465 significantly differentially regulated genes (fold change ≥ 1.0, $P \leq 0.05$) identified in the kidney, 28 are TFs. This quantitatively important regulation of TF's suggests the activation of cellular processes that might mediate the cellular changes/differentiation induced by aldosterone. The output figure of the bibliosphere analysis of the kidney data set acuminate to Nfkb1 (activating form), which is repressed in kidney (CNT/CCD, 1.13 fold) (Figure 27.)

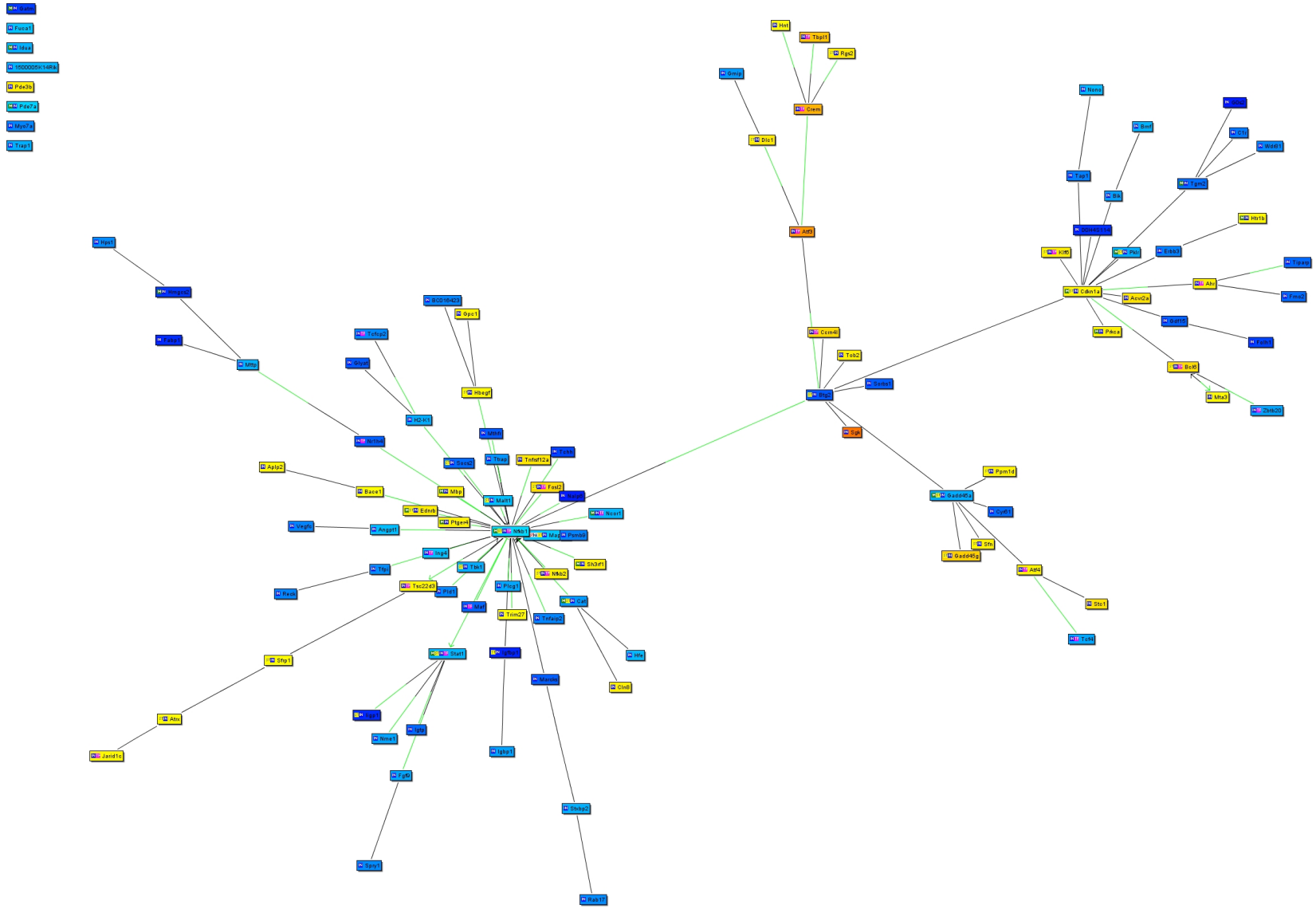

Figure 27. Bibliosphere analysis of kidney (CNT/CCD) data showing the network of transcription factors involved by the stimulation of aldosterone.

Moreover we could find Nfkb2 (inhibitory form) to be induced (1.4 fold). In comparison colon shows no Nfkb regulation, although the number of genes in this group is higher.

Further information is the induction of Cdkn1a in kidney, which appears to be one of the acuminating points in the bibliosphere analysis.

A different set of gene products were found to be regulated by aldosterone in colon. For instance, colon shows regulation of 5 heat-shock proteins (HSPA 1b and 8 up; 4, 4l and 9a down), which was not seen in kidney.

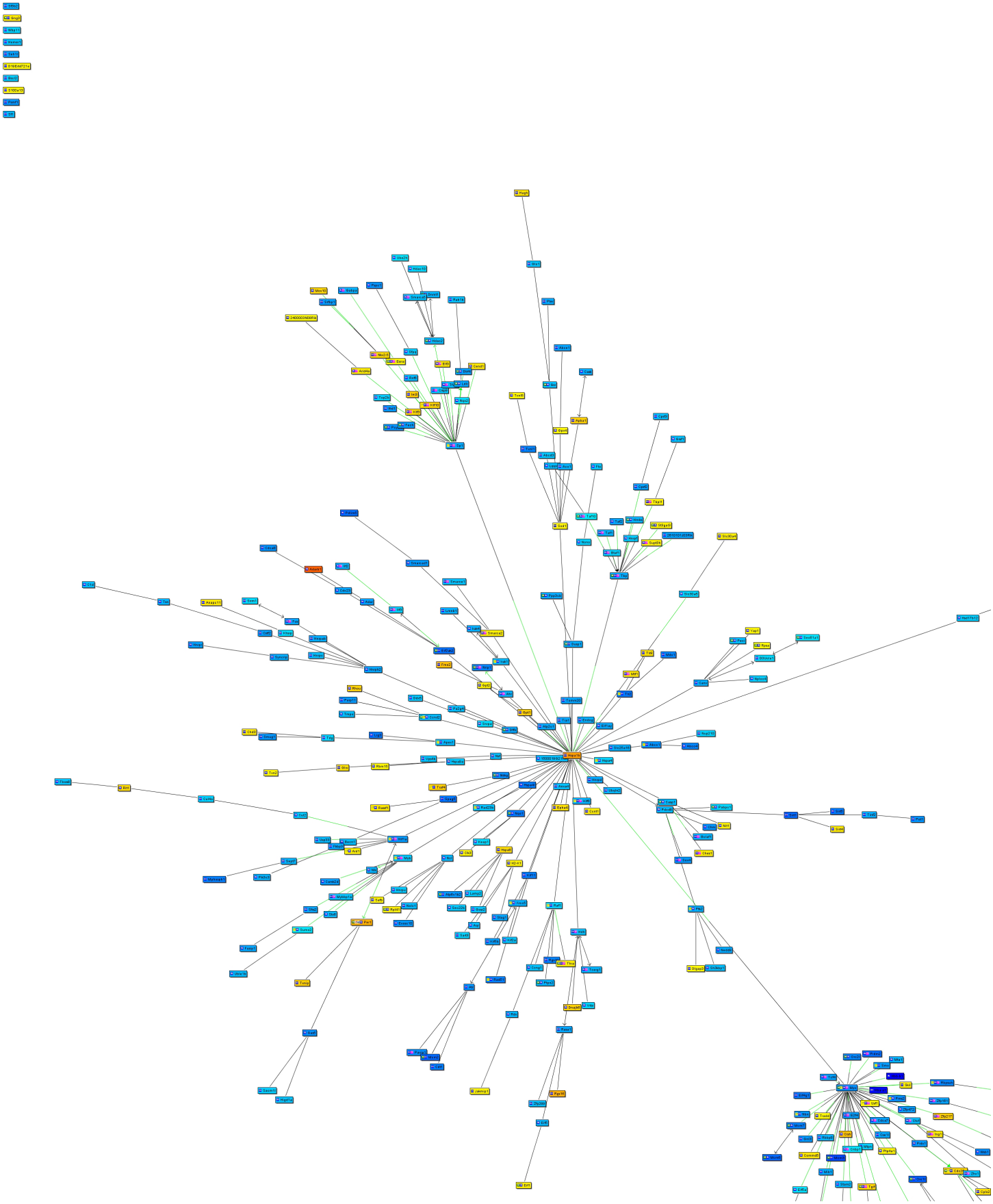

Figure 28. Bibliosphere analysis of colon data showing the network of transcription factors involved by the stimulation of aldosterone.

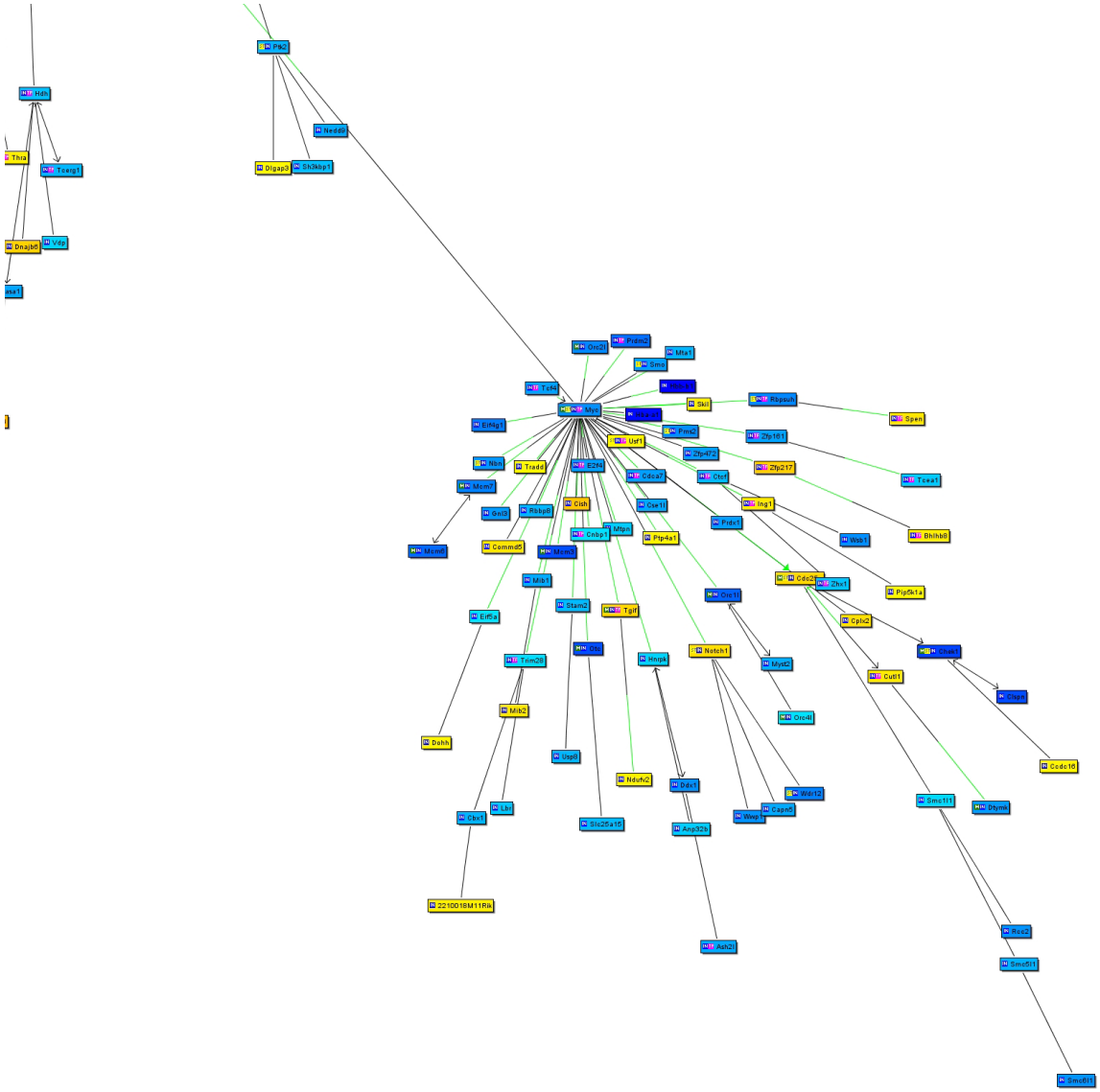

Figure 28.(continued) Bibliosphere analysis of colon data showing the network of transcription factors involved by the stimulation of aldosterone.

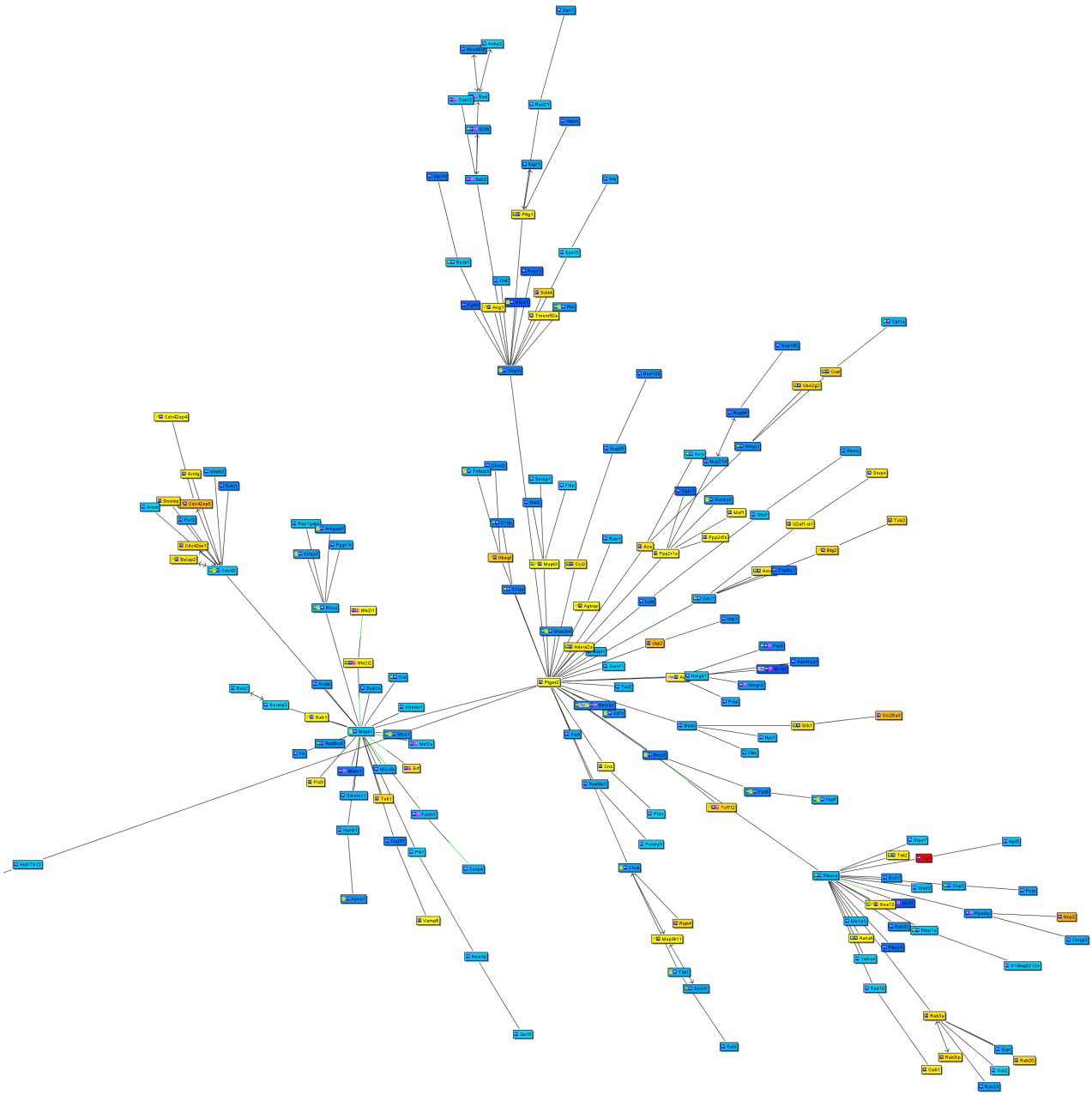

Figure 28.(continued) Bibliosphere analysis of colon data showing the network of transcription factors involved by the stimulation of aldosterone.

Another observation for colon is the repression of Myc (Figure 28.). Additionally; it is striking that unlike in kidney no TF could be found among the top 20 regulated genes. In total 84 TFs out of a total of 1488 significantly differentially regulated genes (fold change ≥ 1.0, $P \leq 0.05$) were found. Other regulated gene products which might play important roles in aldosterone action in the colon are prostaglandine PGES2, MAPK1 and SP1 (Figure 28.).

We then examined the heart microarray data sets in comparison to those of the epithelial tissues from kidney and colon. We also examined a small subset of genes representative for different function by real-time RT PCR. In particular, we found by real-time PCR a modest but significant increase in Sgk1 mRNA of around 1.7 fold (Figure 26.). This confirmed that the heart can be considered as an aldosterone-sensitive tissue in which, similarly to colon and kidney, Sgk1 is activated by short-term aldosterone treatment. In addition to Sgk1, glucocorticoid-induced leucine zipper (GILZ, TSC22 domain family 3), which has been previously described to be induced by aldosterone in other cell types, was also increased as well in heart (around 2-fold).

In addition to SGK1 and GILZ, which are involved in cellular signaling pathways, our microarray identified Rgs2 (Table 5.) another early aldosterone-induced regulatory gene product in mice heart [308]. Rgs proteins play a key role in the regulation of G protein-coupled receptor signaling. G protein-coupled are activated by many extracellular signals that regulate cardiomyocyte growth and differentiation. The heart expresses several Rgs proteins but their individual roles in regulating cardiovascular function are still not clear [309].

Table 5. Differentially expressed genes in mice heart

gene products	Genbank	Gene Symbol	RATIO
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	D67076	**Adamts1**	**1.35**
CCAAT/enhancer binding protein (C/EBP), delta	BB831146	**Cebpd**	**2.65**
nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	NM_010907	**Nfkbia**	**1.417**
regulator of G-protein signaling 2	AF215668	**Rgs2**	**1.557**
serum/glucocorticoid regulated kinase	NM_011361	**Sgk**	**1.515**
TSC22 domain family 3	NM_010286	**Tsc22d3**	**2.137**

Genes involved in inflammatory processes were identified as significantly regulated by short term aldosterone (Table 5.). Among them the nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha (Nfkbia) showed a significant increase of around 1.5 fold. Nfkbia belongs to the PAR1-dependent transcripts, which are known to counter-balance the inflammatory response to PAR activation by limiting prolonged activation of p38 MAPK and increased cytokine production [310]. Another gene product involved during acute inflammation is the CCAAT/enhancer-binding protein delta (C/EBP delta) (Table 5.), which belongs to the family of leucine zipper transcription factors. In mice heart C/EBP delta shows after aldosterone treatment an induction of around 2.6 fold. Additionally, ADAMTS1, a metalloprotease that is highly expressed in the heart and is likely important for the structure or regulation of extracellular matrix, shows a moderate but significant increase of around 1.4 fold.

4. Conclusions

The main objective of the present study was the identification and comparison of early aldosterone-regulated gene products in mouse heart, colon and kidney using different bioinformatic tools to unravel the potential different functions of aldosterone in these tissues. Among the gene products that were found to be regulated by aldosterone were members of regulatory networks that are related to proliferation, differentiation and stress reactions. Those regulatory networks are to some extent common to the different aldosterone target tissues. Regarding kidney, one example is the induction of Cdkn1a, which appears to be one of the acuminating points in the bibliosphere analysis, revealing that aldosterone action directly targets regulatory networks controlling cell cycle progression, terminal differentiation, and apoptosis [311].

Another important gene product among the aldosterone-regulated transcription factors in kidney is NFkB. NF-kappa B comprises a family of transcription factors thought to have a central role in the expression of genes involved in cell mobilization, cell proliferation and cell differentiation, and, hence, in inflammation, repair and fibrosis processes. In particular, NF-kappa B activation has been shown to participate in a number of inflammatory diseases of the kidney that progress to end-stage renal failure [312]. In this context, another interesting regulated transcription factor is myc (Ndrg-1) that is regulated in particular in colon. Ndrg-1 has been identified as a protein involved in the differentiation of epithelial cells [313]. In addition, Ndrg-1 expression can be regulated by androgens and is involved in the pathology of the disease [314].

Aldosterone, in addition to its well-established effects on epithelial target cells, has

been suggested to exert direct effects on the cardiovascular system. The main known effects of aldosterone in classical target tissues are mediated via changes in transcription. On the other hand, only a few genes that are clearly regulated via the MR have been described in cardiac or vascular tissues in cell culture. Among these is Sgk1, which as in renal epithelia [29, 110, 315] is induced by aldosterone in mouse heart tissue. Aside from the four common aldosterone-regulated genes shared by heart and kidney epithelial cells, it seems likely that the majority of the early aldosterone-regulated genes in cardiomyocytes are different from those regulated in epithelia. Importantly, our experiments identified in heart a number of early regulated genes whose role in cardiovascular function has been established but their aldosterone-regulation was previously unknown, as well as other genes that are likely to have a function in cardiac pathology.

In this study we were interested in identifying regulated gene products that have some obvious or putative connection to the regulation of extracellular matrix or cardiac remodeling, for example ADAMTS1, which could play a role in aldosterone's fibrotic effects. ADAMTS1, a metalloprotease and disintegrin, was originally cloned as an inflammation-associated gene [316]. Later it was shown that ADAMTS1 is induced by myocardial infarction in cardiomyocytes and endothelium within the infarct zone [317]. Furthermore, ADAMTS1 can disrupt extracellular matrix remodeling [318], and its overexpression accelerates atherosclerosis and neointima formation [319].

Another group of genes rapidly up-regulated by aldosterone in heart includes genes that have been implicated in inflammation and acute phase reaction. Among those we found Nfkbia and C/EBP delta, both gene products known to play a role in acute inflammation or stress [320]. Further, GILZ, which we found to be

up-regulated by aldosterone belongs to this category of acute-phase proteins [321].

Rgs2, a gene rapidly induced by aldosterone in heart, is involved in signal transduction and regulation of vascular tone. Rgs proteins are multifunctional signaling regulators and exert GTPase-activating protein activities. Many signals that regulate cardiomyocyte growth, differentiation, and function are mediated via heterotrimeric G proteins, which are controlled by Rgs proteins. Rgs proteins are necessary for normal cardiovascular function and are involved in hypertrophy and development of heart failure [322]. Changes in cardiac Rgs2 levels and in G(q/11) signaling produce cardiovascular phenotypes. Furthermore, haploinsufficiency and/or elimination of the Rgs2 gene lead to hypertension in mice [323]. Rgs2 is activated via the nitric oxide-cGMP pathway, which might be part of the mechanism by which it regulates blood pressure [324]. A recent study showed that transgenic mice with activated Gq signaling develop heart failure, but the contractile dysfunction and structural abnormalities improved significantly after termination of the Gq signal [325], suggesting that Rgs2 could be beneficial in heart failure. Such an effect is difficult to reconcile with a predominantly constrictor action of aldosterone and with the current dogma that aldosterone is detrimental in heart failure. On the other hand, induction of Rgs2, and probably other proteins that are cardioprotective, might be part of a compensatory feedback mechanism initiated by aldosterone.

Aldosterone also regulates the expression of several genes that may contribute to the development of cardiovascular damage (fibrosis, vasculitis) under pathological conditions. Blocking aldosterone regulation of these genes by MR antagonists such as epleronone or spironolactone might contribute to the beneficial effects

observed in patients with chronic heart failure. However, it seems that activation of the MR by aldosterone also induces genes, such as Rgs2, that are likely to exert cardioprotective effects and thus might be beneficial in preventing heart failure.

In summary, the analysis of gene regulation by a short-term aldosterone treatment in kidney, colon and heart reveals surprisingly little common regulated gene products between kidney and colon, despite their common function in Na^+ transport. Importantly however, Sgk1 and Usp2-45 were regulated similarly in these two epithelial tissues, stressing their central role for mediating aldosterone action. In all three tissues aldosterone targets, in particular by regulating transcription factors, regulatory networks involved in cell proliferation, differentiation and stress reaction. In that respect, it is interesting to note that there are apparently more common actions in heart and kidney than in colon and kidney. Taken together, these results and their analysis indicate that the short-term transcriptional action of aldosterone is relatively specific for each tissue type.

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Publications, Presentations & Posters

• Publications

C. Wagner, D. Loffing, Q. Yan, N. Schulz, **P. Fakitsas**, M. Carrel, T. Wang, F. Verrey, J. Geibel, G. Giebisch, S. Hebert, J. Loffing;
Mouse Model of Type II Bartters Syndrome. II. Altered Expression of Renal Sodium- and Water-Transporting Proteins;
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Hyperaldosteronemia and activation of the epithelial sodium channel are not required for sodium retention in puromycin-induced nephrosis;
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D. Benke, **P. Fakitsas**, C. Roggenmoser, C. Michel, U. Rudolph, H. Mohler;
Analysis of the presence and abundance of $GABA_A$ receptors containing two different types of alpha subunits in murine brain using point-mutated alpha subunits;
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D. Tsiakas, E. Schwedhelm, F.-M. Gutzki, O. Jahn, **P. Fakitsas** and J.C. Fröhlich;
Enzymatic synthesis of dioxygen-18 labeled 8-epi-prostaglandin F2 alpha and its use in quantitative GC-tandem MS;
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K. Löw, F. Crestani, R. Keist, D. Benke, I. Brünig, J.A. Benson, J.-M. Fritschy, T. Rülicke, H. Bluethmann, H. Möhler and U. Rudolph;
Molecular and neuronal substrate for the selective attenuation of anxiety;
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- **Presentations**

P. Fakitsas, G. Adam, D. Daidié, M. X. van Bemmelen, F. Fouladkou, A. Patrignani, U. Wagner, R. Warth, O. Staub and F. Verrey;
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ZNZ-Symposium, Zürich, Switzerland (Oct. 2000). Institute of Pharmacology and Toxicology, ETH and University Zürich.

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